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**Phosphatidylethanolamine *N*-Methyltransferase Activity—A Risk Factor for
Cardiovascular Disease?**

By

Anna Noga



**A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfillment of the requirements for the degree of Doctor of Philosophy**

Department of Biochemistry

Edmonton, Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Phosphatidylethanolamine N-Methyltransferase Activity - A Risk Factor for Cardiovascular Disease? submitted by Anna Noga in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract:

Phosphatidylethanolamine *N*-methyltransferase (PEMT) is a secondary pathway for phosphatidylcholine (PC) synthesis in the liver. The original goal of this thesis was to examine the role of PEMT derived PC and its contribution to the secretion of very low density lipoprotein particles (VLDL). In order to address this question, we utilized the *Pemt*^{-/-} mouse in both *in vivo* diet studies as well as hepatocyte experiments. After a 3 week high fat/high cholesterol (HF/HC) diet, the male *Pemt*^{-/-} mice demonstrated hepatic triglyceride (TG) accumulation, depleted plasma TG levels as well as reduced secretion of apolipoprotein (apo) B100 containing particles, all indicative of an impairment in VLDL secretion. Female *Pemt*^{-/-} mice maintained normal VLDL secretion with both chow and HF/HC dietary conditions, however these animals had decreased levels of PC and cholesterol in the high density lipoprotein fraction. Experimentation using primary hepatocytes isolated from the *Pemt*^{-/-} males confirmed that in the presence of a high fat challenge, both TG and apoB100 secretion was impaired.

Since we determined that PEMT derived PC is required for VLDL secretion in a gender and dietary dependent fashion, we were intrigued as to whether or not PC metabolism was regulated by these 2 variables. Enzymatic assays of *Pemt*^{+/+} males and females on chow or HF/HC diets suggest that total PEMT protein mass is not regulated by either gender or diet. Western blots did suggest that PEMT is distributed intracellularly in a gender specific fashion. As well, enzymatic assays of cytidyltransferase, the rate limiting step of the predominant PC biosynthetic pathway (Kennedy pathway) suggest that male mice rely more heavily on the Kennedy pathway than females. *In vivo* radiolabel injections of a PEMT substrate into the *Pemt*^{+/+} mice

confirmed that females generally rely more heavily on the PEMT pathway for PC synthesis, compared to male mice.

Finally, homocysteine (Hcy) is a byproduct of the PEMT pathway, and it is well established that this molecule is toxic whenever it accumulates either intracellularly, or in the plasma. We utilized the *Pemt*^{-/-} mouse in both *in vivo* and hepatocyte experiments to determine whether or not this enzyme contributes significantly to plasma Hcy levels. Our studies confirm that PEMT activity contributes up to 50% of total plasma Hcy in mice. Preliminary results obtained with the hepatocytes also confirmed that PEMT activity contributes significantly to exported Hcy pools.

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Although this thesis is a culmination of 6 years of hard work (and some play) on my part, its completion would not have been possible without many key people. First and foremost I would like to acknowledge my supervisor Dr. D.E. Vance who allowed me to work in his lab and essentially gave me the freedom to aim the work in the direction that I chose. I would also like to thank him greatly for his patience, guidance and encouragement over the last few years. I would also like to thank the other members of my supervisory committee, Dr. Charles Holmes and Dr. Richard Lehner. I would especially like to thank Richard for the time he took to teach me many techniques and essentially guide me in my first year of graduate studies, when he was working as a post-doc in our lab.

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In addition to the friends I made in the lab, I have made many other friends with people on our floor. Many a good time was had either working late at night in the lab, or going out to Avenue, ReBar, Second Cup, parties and so on. Everyone over the years either helped me by proofreading my work, getting me out of computer jams, running HPLCs for me, killing my rats, listened to me blow steam, etc... You all know who you are!! ☺ Finally, I would like to acknowledge our collaborators, Dr. J. Brosnan and Lori Stead who actually made the homocysteine project a possibility.

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Abbreviations

ABCA-1	ABC Transporter A-1
AdoHcy	S-Adenosylhomocysteine
AdoMet	S-Adenosylmethionine
Apo	apolipoprotein
AADA	arylacetamide deacetylase
CBS	cystathionine β -synthase
CD	choline deficient
CE	cholesteryl ester
CETP	cholesterol ester transfer protein
CHO	chinese hamster ovary
CK	choline kinase
CM	chylomicron
CPT	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CT	CTP: phosphocholine cytidyltransferase
Cys	cysteine
DAG	diacylglycerol
ER	endoplasmic reticulum
Hcy	homocysteine
HDL	high density lipoprotein
HF/HC	high fat/high cholesterol
HPLC	high performance liquid chromatography
IDL	intermediate density lipoprotein

kDa	kilodalton
LCAT	lecithin cholesterol acyltransferase
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
Lp(a)	lipoprotein (a)
LPC	lyso-phosphatidylcholine
LPL	lipoprotein lipase
Lys	lysine
MAG	monoacylglycerol
Met	methionine
MAM	mitochondrial associated membrane
MTP	microsomal triglyceride transfer protein
n-3	omega 3
PC	phosphatidylcholine
PDI	protein disulfide isomerase
PDME	phosphatidyl dimethylethanolamine
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine <i>N</i> -methyltransferase
PG	proteoglycan
PI	phosphatidylinositol
PMME	phosphatidyl monomethylethanolamine
PS	phosphatidylserine
SM	sphingomyelin

SR-B1	scavenger receptor B-1
SREBP	sterol regulatory element binding protein
TG	triacylglycerol
TGH	triglycerol hydrolase
TLC	thin layer chromatography
VLDL	very low density lipoprotein

1

Chapter 1

Introduction

1.1 Cardiovascular Disease

Cardiovascular disease is the predominant cause of mortality in the developed world (1). The prevalence of this disease state within the population is due to the increased exposure to a number of risk factors including a high fat/high cholesterol diet (HF/HC), obesity, smoking, diabetes and genetic predisposition within subpopulations (2, 3). The development of atherosclerotic plaques on the artery walls results in blockage of blood flow to a particular organ. In most cases the organs affected are either the heart muscle, or the brain (stroke).

Atherosclerotic plaques develop as a result of lipid deposits and activated inflammatory cells accumulating within the intima of the artery wall (1). Although there are a number of risk factors that contribute to the development of the atheroma, its synthesis is ultimately dependent on an overabundance of apolipoprotein B (apoB) containing lipoproteins, primarily low density lipoproteins (LDL) in the plasma (4-6). In animal studies, even in the presence of other risk factors, atherosclerotic lesions do not develop unless levels of apoB containing lipoproteins are also high (4, 7).

ApoB containing lipoproteins are responsible for transporting lipids from the intestine, as well as the liver to the peripheral tissues (8). Lipids play a number of important roles in mammals. For example, lipids such as triglycerides (TG) serve as an efficient source of energy for the body (9). Cholesterol is required for embryonic development (10), cellular membrane structure (11) and the synthesis of steroid hormones (12). Finally, phospholipids are also required for membrane structure (11), and cell signaling functions (13). However, the hydrophobic nature of TG and cholesteryl esters (CE) requires that they be packaged into a soluble lipoprotein for transport in the plasma. A number of different lipoproteins have several functions in the organism (8). This very efficient physiological system for distribution of lipid to the various tissues more than likely evolved for times of food shortage. In industrialized countries starvation is no longer a major problem and the diets are saturated with fat and cholesterol. This increase in dietary fat intake results in an overall increase in plasma apoB containing lipoproteins and ultimately their contribution to the development of atherosclerotic plaques.

1.2 Development of the Atheroma

The first determinant for the development of the plaque is the entry of LDL into the arterial intima through the monolayer of endothelial cells. LDL is able to enter and leave the artery wall when the endothelium and intima are normal (14). Permeability of the endothelium for LDL penetration is increased when the endothelium is injured (15-17) or activated as a result of factors such as sheer stress, local adherence of platelet cells, lipoprotein aggregation and oxidation as well as smooth muscle cell alterations (4). Furthermore, the atherosclerotic lesion is primarily composed of lipid-enriched macrophages (1). Endothelial injury results in the adherence of circulating monocytes and T-lymphocytes to the site, entry into the arterial wall via chemotaxis, and uptake of trapped LDL (1). The "response to injury" hypothesis (18-20) suggests that modification of the endothelium is a required step in the development of the early atherosclerotic lesion. However, the observation of fatty deposits occurring under an intact endothelium suggests that although endothelial injury may accelerate plaque development, it is not a required step (4).

Another hypothesis suggests that the major pathological event for plaque development is retention of atherogenic lipoproteins within the intima (4, 14, 21, 22). Under ideal physiological conditions, the rate of entry of LDL into a normal intima is the same as the number of particles lost either due to degradation, processing by the muscular media, or exit back into the plasma (4, 14, 17, 23). However, when endothelial permeability is increased (14-17), or plasma LDL concentrations are very high due to the onset of hypercholesterolemia (24), entry of these atherogenic particles through the endothelium exceeds exit and the particles are retained within the intima. Components of the atherogenic lipoproteins trapped in the intima then stimulate the expression of cell adhesion molecules that continue to recruit monocytes/macrophages and leukocytes, which then enter the arterial wall and take up the particles (25, 26).

The intima is largely an acellular lamina between the endothelium and the smooth muscle cell media. This lamina is largely filled with extracellular matrix consisting of collagens, elastin fibers as well as various proteoglycans (PG) (14). It is hypothesized that the LDL particle can bind to the negatively charged PGs by way of basic residues

found in the apoB100 protein, which surrounds the lipoprotein particle (residues 3145-3157 and 3359-3367) (14, 27-30). Once trapped within the intima, the LDL particle is susceptible to modification by a number of resident factors that enhance the atherogenicity of the particle (14).

Firstly, irreversible binding of the particle to PGs alters the particle surface such that apoB100 is more susceptible to proteolytic hydrolysis (14, 31). Also, secretory phospholipase A₂ is resident in human lesions and breaks down the LDL phospholipids (32-35). The lyso-phosphatidylcholine (LPC) produced is a potent mitogen for smooth muscle cells (36, 37), and it induces the expression of cell-adhesion molecules on the endothelium (25). Thirdly, sphingomyelinase activity in the arterial wall (38, 39), and on the LDL particle (40), could induce LDL aggregation and generate the lipid messenger ceramide, which also may induce changes in the surrounding cells (41). Finally, long retention times of LDL particles in the intima allow for oxidation of the outer monolayer phospholipids that surround the neutral lipid core of the particle (42-44).

Circulating monocytes/macrophages influx into the intima and take up these modified LDL particles through a number of scavenger receptors. Type A scavenger receptors I and II, and the type B scavenger receptor CD 36, are hypothesized to be the main receptors in humans which recognize oxidized LDL (45). The scavenger receptor pathway only recognizes modified and not native LDL (46). There is no feedback system to control the quantity of cholesterol taken up by the macrophages, in contrast, CE accumulation stimulates the expression of CD 36 to accelerate uptake of modified LDL (47). The cholesterol taken up is acylated and stored in neutral lipid droplets as CE (48). The macrophages become full of lipid and at this stage are considered foam cells (1, 46). Lesion formation occurs when many of these foam cells accumulate under the endothelium and form a fatty streak (1, 46) (Fig. 1.1 A).

Eventually, the foam cells become so CE laden that they become necrotic, releasing their internal contents (1, 49). The necrotic contents and storage pools of CE cause injury to the endothelium and therefore further increase LDL permeability and attract more monocytes, continuing the atherogenic cycle. Furthermore, platelet derived

Figure 1.1: Development of an Atherosclerotic Lesion

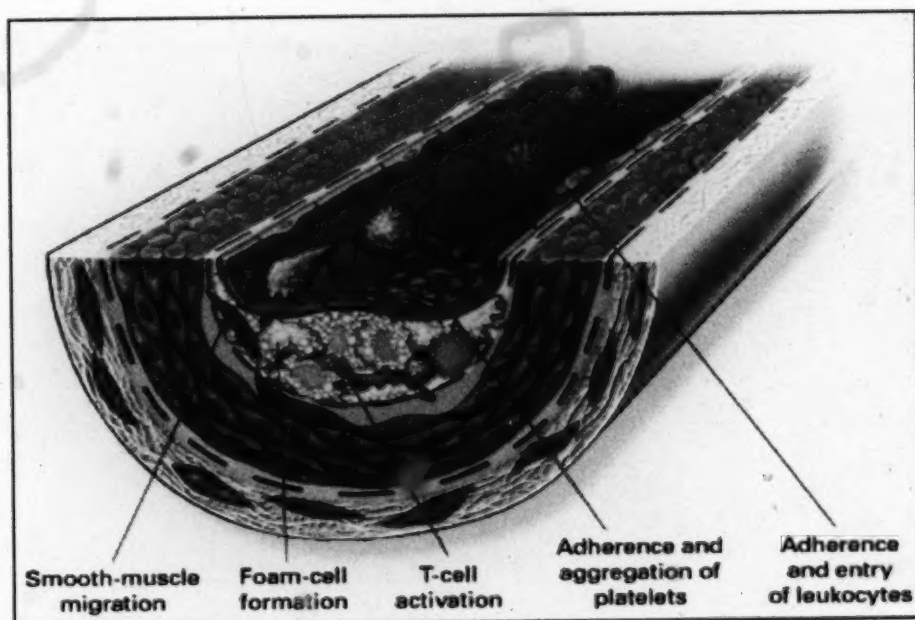
A. Fatty Streak Formation: In the fatty streak, a number of foam-cells have accumulated within the intima of the vascular wall. At this stage smooth muscle cells begin to migrate into the intima due to stimulation by a number of growth factors that are recruited to repair damage to the wall inflicted by the accumulation of lipid. The atherogenic lipoproteins that accumulate in the vascular wall stimulate the expression of a number of cell adhesion molecules that recruit additional inflammatory cells such as platelets and leukocytes.

B. Advanced Atherosclerotic Lesion: Many foam cells have accumulated within the intima and some have become so engorged with CE they have become necrotic. The release of the CE from these cells causes additional damage to the vascular wall. The smooth muscle cells that migrated into the intima over time secrete collagen, elastin and PGs resulting in a thickened intima, as well as a fibrous cap, which covers the lesion. As well, more inflammatory cells and lipoproteins are attracted to the lesion since cycles of injury to the vascular wall continues to promote adhesion molecule expression and infiltration through the endothelium.

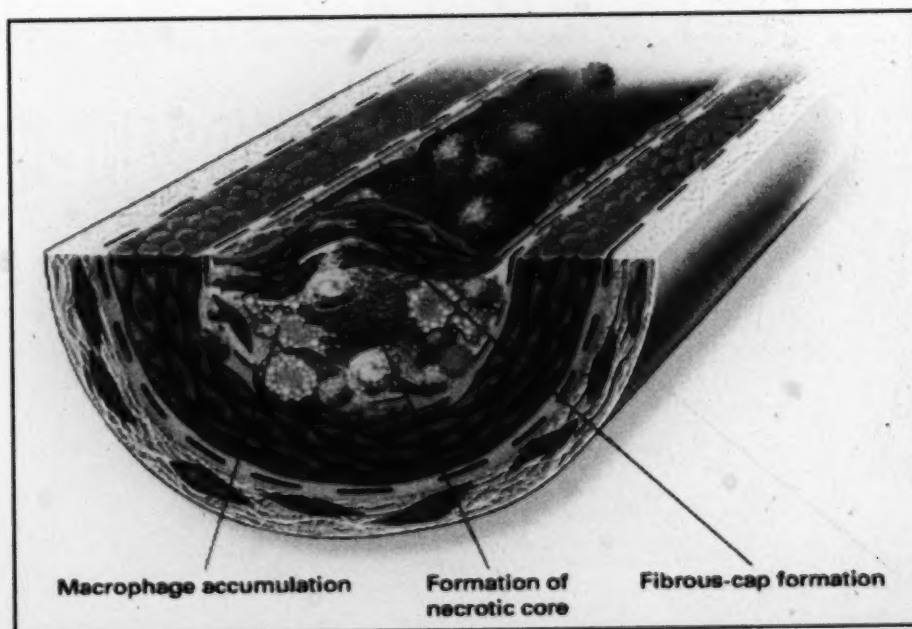
C. Rupture of the Fibrous Plaque: Eventually, the atherosclerotic lesion may become so large that it blocks efficient blood flow. In addition, advanced lesions may also bear signs of calcification and hemorrhaging. However a cardiovascular event may also occur if the fibrous cap covering the atherosclerotic lesion becomes unstable. The continuous release of proteolytic enzymes by activated macrophages results in thinning of the fibrous cap, and rupture, resulting in thrombosis.

(figure taken from reference 1)

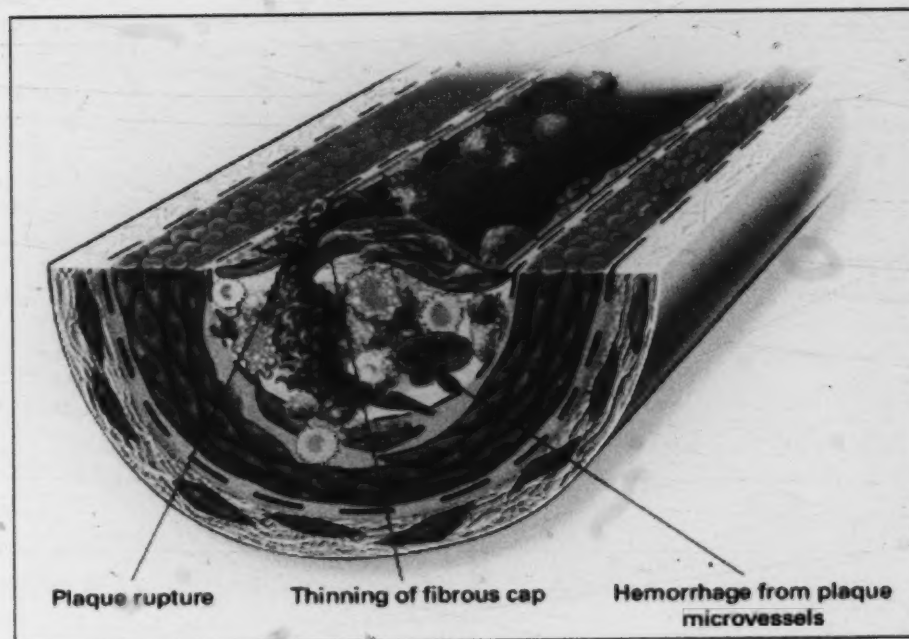
A.



B.



C.



growth factor is also recruited to repair the injury and this stimulates the proliferation of the smooth muscle cells (1, 49).

Over time the lesion becomes larger with more lipid deposition and smooth muscle cell growth. The smooth muscle cells migrate from the media into the intima and secrete collagen, elastin and PGs thickening the intima and forming a fibrous cap to stabilize the plaque (1, 14, 49) (Fig. 1.1 B). As a consequence, the artery wall becomes more rigid resulting in impaired blood flow. An advanced lesion is a combination of smooth muscle cells, connective tissue, cholesterol and necrotic cells with calcification and potential bleeding (1). These plaques grow until blood flow is stopped, or they rupture and inhibit blood flow in another region of the body (1, 49) (Fig. 1.1 C).

1.3 Cardiovascular Disease Risk Factors

1.3.1 High LDL/HDL Ratios: As mentioned above, there are a number of different lipoproteins that serve several functions in the body with regards to lipid distribution. All lipoproteins are spherical components consisting of a soluble outer monolayer of phospholipids, cholesterol and apolipoproteins and an inner core of neutral lipid. Lipoproteins are classified based on their size, buoyant density, lipid composition and associated apolipoproteins (8). Generally, the different lipoproteins can be classified into 2 functional categories (8).

	CM	VLDL	IDL	LDL	HDL
Density (g/ml)	<0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.21
Diameter (nm)	75-1200	30-80	25-35	18-25	5-12
Composition (% dry weight)					
Protein	1-2	10	18	25	33
TG	83	50	31	9	8
Cholesterol & CE	8	22	29	45	30
Phospholipid	7	18	22	21	29
Apoproteins	A1, A2 B-48 C1, C2, C3 E	B100 C1, C2, C3 E	B100 C1, C2, C3 E	B100	A1, A2 C1, C2, C3 E

Table 1.1: Lipoprotein Classification

A summary of the different classes of lipoproteins and their composition. Chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and LDL constitute the apoB containing class of lipoproteins. Other than LDL, these lipoproteins transport primarily TG. There are 3 classes of high density lipoproteins (HDL) which compose the apoAI containing class of lipoproteins. The neutral lipid core of this class of lipoproteins is primarily CE (taken from reference 54).

A) ApoB Containing Lipoproteins: ApoB containing lipoproteins are responsible for transporting intestinal and hepatic derived lipids to the rest of the tissues (8). CMs are the largest lipoproteins and are secreted from the intestine (8). These lipoproteins are responsible for the packaging and distribution of dietary fats, and fat-soluble vitamins to the periphery (8, 50). The predominant apolipoprotein of CM particles is apoB48, a post-transcriptionally edited variant of apoB100, which in humans is expressed only in the intestine (8, 50). The CM particle also has a number of the smaller apoCII proteins associated on the surface (8). ApoCII is required for the association of this particle with lipoprotein lipase (LPL), the enzyme responsible for the progressive loss of TG in the particle (8, 51). LPL is usually found on the capillary endothelial surface of adipocytes and skeletal muscle via an association with cell surface heparin (8, 51). LPL rapidly hydrolyzes the TG within the neutral core releasing free fatty acids and 2-monoacylglycerol (MAG) which are then taken up by the cell (8). Once the CM has decreased to a certain size, it can no longer support the apoCII proteins and they are lost to HDL particles (8, 51). At this point apoE facilitates uptake of the CM remnant into the liver via association with the LDL receptor (LDLR) and the LDLR related protein (52, 53) (Fig. 1.2).

VLDL are TG rich particles that contain apoB100, several molecules of apoCII as well as apoE apolipoproteins (8, 54). The liver secretes these particles with the purpose of transporting hepatically synthesized TG and cholesterol to the other tissues (8, 54). VLDL are also processed by LPL (8, 51) (Fig. 1.2). With the hydrolysis of TG, the particle size and density decreases to the IDL stage of lipoprotein metabolism. IDL can undergo further lipolysis via LPL, however, eventually size constraints result in the loss of all apoCII molecules (8). Otherwise, the LDLR recognizes apoE on the IDL particle and takes it up by endocytosis (8).

IDL particles not taken up by the LDLR are further processed to smaller, more CE dense particles known as LDL. Conversion of IDL to LDL involves the loss of 80-90% of core TG levels, and a 50% increase in total CE (8). This processing is performed by either further lipolysis, or the CE transfer protein (CETP), which catalyzes the transfer of CE from the HDL to the IDL particles in exchange for TG (8, 51). The TG transferred

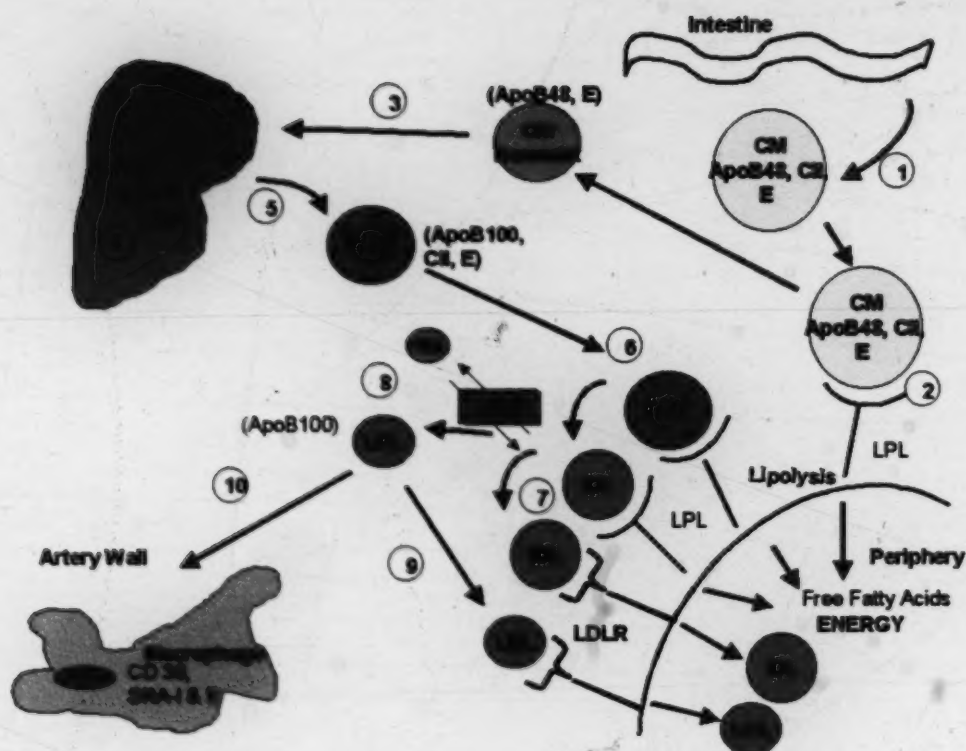


Figure 1.2: Summary of ApoB Containing Lipoprotein Metabolism

1. Dietary fats are packaged into large soluble CM particles in the intestine that are secreted into the bloodstream. 2. The CM particles are rapidly metabolized because their large size accommodates many apoCII molecules that mediate lipolysis of core TG by LPL. 3. After several lipolysis steps, size constraints result in loss of all apoCII molecules. The CM remnant travels to the liver where 4. it is taken up either by the LDLR or the LDL receptor related protein (LRP). 5. TG and CE synthesized in the liver are packaged and secreted as VLDL particles. 6. VLDL particles in the bloodstream come into contact with LPL and undergo lipolysis to become IDL particles. 7. IDL can be taken up by the liver or peripheral tissues via endocytosis that is mediated by the LDLR. 8. IDL not taken up by the LDLR can be further processed down to LDL particles. The IDL either undergoes further lipolysis, or it exchanges its TG for HDL derived CE. This transfer is mediated by CETP. 9. LDL particles can be endocytosed by the LDLR located in the peripheral tissues or at the liver. 10. Excess circulating LDL particles not taken up, are susceptible to accumulating in the arterial wall. Once trapped in the intima, these particles are modified and taken up by scavenger receptors on macrophage cells.

to the HDL is then rapidly broken down by hepatic lipase (8, 51). At this stage, the LDL only has the apoB100 protein remaining but it is now in a conformational state that it too is recognized by the LDLR (55).

The function of the LDLR is to provide the cell with necessary cholesterol that it may not necessarily be able to maintain by *de novo* synthesis (53). However, there is a feedback mechanism that prevents the accumulation of cholesterol in the cell. The sterol regulatory element binding protein (SREBP) family of transcription factors are transmembrane proteins localized at the endoplasmic reticulum (ER) membrane in their inactive form (12, 56). These proteins are associated with the SREBP cleavage activating protein which is able to sense the levels of cholesterol in the membrane (56). When cholesterol levels are low the SREBP cleavage activating protein mediates the mobilization of SREBP to the Golgi where it is proteolytically cleaved to its active form (12, 56). The active form translocates to the nucleus where it binds to SRE sites in the promoters of a variety of genes that regulate the metabolism of cholesterol and other lipids (12, 56). When sterol levels are low, the activation of these transcription factors results in an increase in cholesterol synthesis as well as an increase in the number of LDLR molecules on the cell surface (12, 56). Excess cellular cholesterol means that the transcription of LDLR is downregulated and so IDL and LDL particles are unable to reach their destinations. With nowhere else to go, excess LDL circulating in the plasma is available for penetration through the arterial endothelium and retention in the intima.

B) ApoA1 Containing Lipoproteins: ApoA1 containing lipoproteins constitute all the different classes of HDL (8). Unlike the apoB containing particles, the neutral lipid core of HDL particles is predominantly CE and not TG (8). HDL particles are hypothesized to perform reverse cholesterol transport of cholesterol from the peripheral tissues back to the liver (57). The liver uses this cholesterol primarily to produce bile that is secreted and used for digestive purposes (12). Conversion into bile acids is the primary means for the body to remove cholesterol (12).

ApoA1 is initially secreted into the plasma by the liver or intestine, loosely bound to apoB containing particles (Fig. 1.3) (8). Once in the plasma the apoA1 dissociates

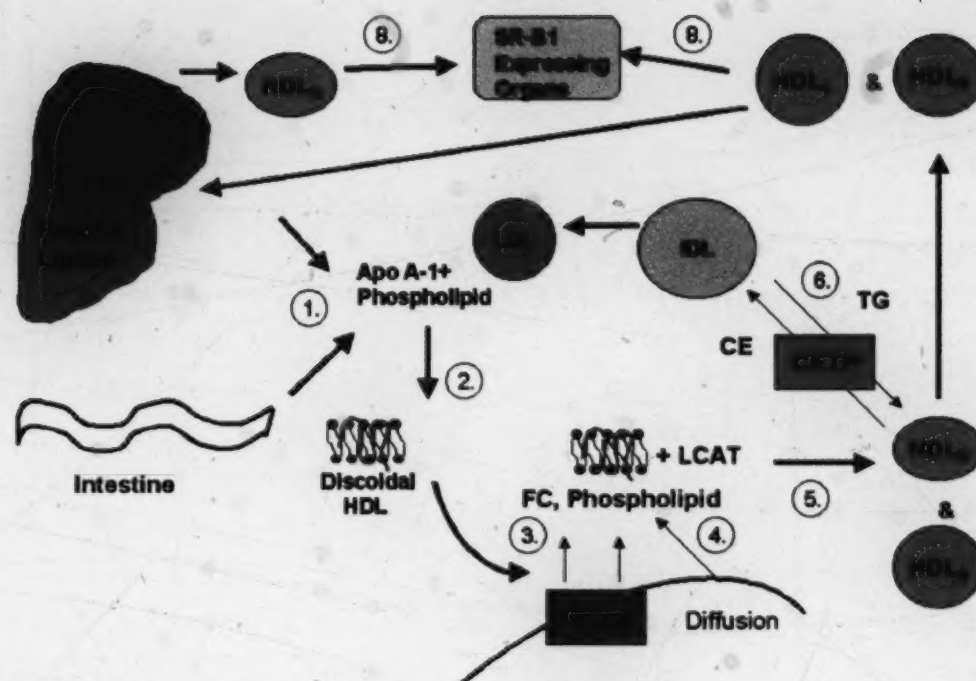


Figure 1.3 Summary of HDL Metabolism

1. Free apoA1 is secreted from the liver or the intestine. 2. ApoA1 associates with phospholipid to form a discoidal particle. Association of apoA1 with phospholipid may require the ATP binding cassette-1 transporter (ABCA-1). 3. The discoidal HDL particle mediates cholesterol and phospholipid efflux from cells via its interaction with ABCA-1. LCAT esterifies the free cholesterol reaching the HDL particle, forming a neutral lipid core. 4. Although most lipid efflux to HDL is mediated by ABCA-1, there is some evidence suggesting that some cholesterol reaches the HDL particle by diffusion. 5. Depending on the degree of efflux, as well as LCAT activity, the HDL particle is now spherical with a CE core. 6. Transfer of TG from IDL to HDL via CETP activity increases the size and buoyant density of the particle. 7. The HDL TG is metabolized by hepatic lipase (HL) and the particle decreases in size. 8. HDL particles of all sizes interact with the scavenger receptor type B1 (SR-B1), which mediates CE and phospholipid uptake. SR-B1 is located in the liver as well as steroidogenic tissues such as the adrenal gland. SR-B1 does not endocytose the particles but simply removes the lipid. Therefore the apoA1 containing particle is still in the circulation, ready to mediate more cholesterol efflux from areas such as the fatty streak.

from these particles and becomes bound to phospholipid in the form of a discoidal particle (8). This pre- β HDL particle then induces efflux of cholesterol from surrounding cells, including the macrophage foam cells in the fatty streak (8). Efflux of free cholesterol from the plasma membrane of cells to the HDL particle is believed to occur through two mechanisms, diffusion and protein mediated efflux (58). Lecithin: cholesterol acyltransferase (LCAT) in the plasma facilitates the diffusion of cholesterol from the plasma membrane of the cell to the HDL particle (58). It functions to transfer an acyl group from phosphatidylcholine (PC) to the free cholesterol molecule to form CE and LPC (8). In this way, LCAT is hypothesized to maintain the free cholesterol gradient from high in the plasma membrane to low on the HDL particle surface (58). It also is required to make the neutral CE core in the HDL particle, facilitating the increased total cholesterol transport since free cholesterol is limited to the outer monolayer of the particle (8, 58).

Efflux of cholesterol by means of diffusion is a very minor means of transferring cholesterol from the cell to the HDL particle. Although the mechanism is still not well understood, the majority of phospholipid and cholesterol efflux to the HDL particle requires the expression of the ABCA-1 transporter on the surface of the cells (58, 59, 61). Tangier patients lack a functional ABC-1 transporter and these people are HDL deficient (58-61).

As the HDL particle core fills with CE via the actions of LCAT, its size and shape change such that it becomes larger, spherical and the density decreases (8, 58). There are 3 main classes of HDL based on their buoyant densities, HDL-1, -2 and 3 with 3 as the smallest and most dense (8). As described previously, CETP transfers CE from the HDL particle to IDL in exchange for TG also resulting in an increased size and buoyancy of the HDL particles (8). Through the activity of CETP, HDL also contributes to the CE pool in the apoB containing lipoproteins facilitating the formation of LDL from IDL (8). In this way, HDL also assists in the distribution of cholesterol to the organs that require it the most. SR-B1 is a member of the scavenger receptor family and it mediates selective cholesterol uptake from HDL (62). SR-B1 is expressed on the surface of a number of steroidogenic organs as well as the liver (63). In addition to selective cholesterol uptake, SR-B1 has also been recently shown to mediate phospholipid uptake from HDL by

human monocytes and baby hamster kidney cells (64). The cholesterol taken up by this receptor is either utilized for the production of steroid hormones (62) such as estrogen and testosterone, or it is specifically targeted for bile secretion (65).

1.3.1 High LDL/HDL Ratios: Simply based on the above described functions of apoB and apoA1 containing lipoproteins it is clear that apoB lipoproteins contribute to the development of atherosclerosis and HDL contributes to the removal of cholesterol from these lesions (8). CM, VLDL and IDL particles are generally too large to pass the endothelial monolayer on the arterial wall into the intima (14). Only the CE rich LDL and HDL can reach the intima to proceed with their opposing effects on the atherosclerotic fatty streak (14).

Not only do the beneficial effects of a high concentration of HDL seem logical, it has also been shown by a number of human studies. In most cases, a high plasma HDL concentration has a protective effect against the development of cardiovascular disease (66-69). Because HDL is believed to protect one from the development of atherosclerosis, it is frequently named "good cholesterol", while the pool of LDL cholesterol is termed "bad cholesterol".

Genetic defects that severely elevate the levels of LDL in the plasma usually result in the early development of cardiovascular disease (70). For example, familial hypercholesterolemia is a common genetic defect occurring at a heterozygosity rate of 1 in 500 people, and a rate of 1 in 1 million for homozygotes (53). In this disease state a genetic mutation in the LDLR makes it unable to recognize and take up LDL particles (53). Interestingly, patients afflicted with genetic mutations that critically lower HDL levels such as Tangiers disease (71) and LCAT deficiency, do not necessarily have an increased risk for developing early onset cardiovascular disease (58). In the case of Tangier patients, this may be a direct result of also abnormally low plasma LDL concentrations (58, 71)

1.3.2 Diet: A large regulatory determinant of the LDL/HDL ratios is dietary fat and cholesterol uptake. As mentioned above, mammalian cells are capable of synthesizing cholesterol *de novo* (12). Since cholesterol is such an important molecule

with regards to maintenance of cellular membrane structure (11), physiology was designed to also distribute dietary cholesterol to the cells to confirm a constant supply of cholesterol for all cells (53). However, the diets of industrialized countries now have an unnecessarily high concentration of cholesterol. Studies in rodents have shown that feeding of a high cholesterol diet results in the secretion of VLDL particles with a higher concentration of CE (72-74). As mentioned previously, the SREBP family of transcription factors tightly regulates intracellular cholesterol levels, and in mice, activation is downregulated by excess dietary cholesterol (75). In addition, cholesterol is not catabolized and there are limited avenues for removal of this molecule from the body such as sloughing of skin and loss of biliary cholesterol during excretion (12). In combination, these observations suggest that continuous uptake of cholesterol through the diet results in a large LDL cholesterol pool that is not taken up because the LDLR is downregulated. With no efficient pathway to remove this excess cholesterol pool from the organism, these LDL particles remain in the circulatory system and are susceptible to accumulation in the artery walls.

An additional cardiovascular disease risk associated with excessive dietary intake of fat is known as the post-prandial state (51, 76). Western society spends much of its time between the consumption of regular meals, unlike third world countries where more people are found in the fasted state (76). Studies have shown there to be an accumulation of TG rich lipoproteins in the plasma after fat intake. The mechanism for this is believed to be the delayed lipolysis of VLDL particles (76). VLDL is continuously secreted from the liver and it undergoes continuous delipidation by the actions of LPL. However, CM particles compete with VLDL particles for LPL binding sites (51, 76). The interrupted delipidation of VLDL, by the regular release of CM particles (77, 78), results in more acceptor particles of CE by the activity of CETP (76). The result is an accumulation of CE rich, apoB100 containing particles in the plasma (76, 79).

Both obesity and lipotrophy results in a predisposition to the development of type II diabetes (80). Type II diabetes occurs when the insulin receptor on cells becomes insensitive to the actions of insulin (80). One major problem associated with type II diabetes is a hypertriglyceridemia with high plasma concentrations of VLDL and small LDL, and low concentrations of HDL (81). Secretion of insulin into the circulation

stimulates energy storage resulting in the synthesis and storage of TG, primarily in the adipocyte (80, 82). In the absence of insulin, or an insulin response, the fat stores in the adipocyte are hydrolyzed by the activity of hormone sensitive lipase (80, 83). The free fatty acids released into the plasma are an important substrate for TG synthesis in the liver, which is subsequently packaged into VLDL and secreted. Loss of insulin sensitivity results in an accumulation of free fatty acids in the plasma and continuous TG production in the liver for VLDL secretion (80). Furthermore, insulin signaling reduces the expression of microsomal transfer protein (MTP), a protein essential for the assembly of apoB containing lipoproteins (84, 85). With the onset of insulin insensitivity, MTP expression is not inhibited in response to the dietary intake of sugars and therefore production of VLDL is not impaired as it would be normally (85).

Finally, increasing evidence suggests that dietary omega 3 (n-3) fatty acids exert a protective effect against the development of cardiovascular disease (86). The two principal n-3 fatty acids are eicosapentaenoic and docosahexaenoic acids and they are mainly found in fish and fish oils; foods not eaten frequently in a typical North American diet (86). Most human studies have shown that an increase in fish or fish oil consumption resulted in significant decreases in mortality due to cardiovascular disease, as compared to controls (87-89). Dietary intake of these fatty acids is known to decrease plasma TG levels (90), and studies using rat hepatocytes and a rat hepatoma cell line showed that n-3 fatty acids stimulate the degradation of apoB (91, 92). The reduced plasma TG levels observed in humans could also be due to the enhanced post-prandial clearance of CMs and CM remnants stimulated by n-3 fatty acids (86, 93). Dietary intake of fish oil also has beneficial effects beyond changing plasma lipid profiles (86). N-3 fatty acids have been shown to improve vascular function by increasing vasodilation and therefore decreasing blood pressure (86). Dietary fish oils also reduce the expression of intercellular adhesion molecule 1 and scavenger receptors type A-I, II on murine macrophages (94) and inhibit platelet aggregation in humans (95).

1.3.3 Lipoprotein (a): Elevated levels of plasma Lp(a) are recognized as an independent risk factor for the development of cardiovascular disease (96). Lp(a) resembles a LDL particle in its lipid composition (96). However, Lp(a) is unique in that

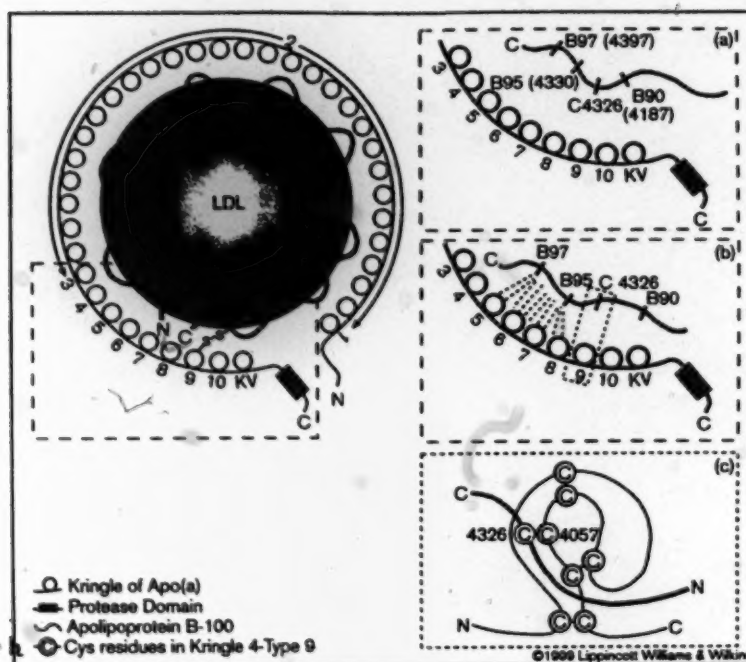


Fig. 1.4: Structure of Lp(a) Particle

Lp(a) consists of a LDL particle with an apo(a) protein covalently linked to apoB100. Apo(a) is very homologous with plasminogen, especially in the KV region and the inactive protease. There are nine unique KIV motifs (KIV type 1 and KIV type 3-10). The KIV type 2 motif can vary in copy number, resulting in massive differences in the size of apo(a) between individuals. (a) Apo(a) associates with apoB100 on the LDL particle first in a non-covalent fashion. Lysine binding sites found in KIV types 5-8 interact with the C-terminal region of apoB100. (b) Next, a disulfide bridge is formed between the last Cys4326 in apoB100 with Cys4057 within the KIV type 9 motif. (c) Finally, the structure of a krigle motif consists of a loop structure that is formed via 3 disulfide bridges. Here, the covalent bridge between KIV type 9 and apoB100 is also demonstrated (figure taken from reference 96)

it has covalently attached to the apoB100 protein, the plasminogen homologue, apolipoprotein (a) (96) (Fig. 1.4).

The sequence of plasminogen contains 5 kringle motifs (KI-V) which are cysteine rich protein structures (96, 97). The cysteines within each kringle domain form 3 intramolecular disulfide linkages forming a tri-loop structure (Fig. 1.4c) (96). The kringle domains for apo(a) resemble those in plasminogen except in the region of KIV where the type 2 repeat (there are altogether 10 different types of repeats within KIV) can be multiplied anywhere from 8 to 43 times (96, 97). This results in a protein that can vary in size from 300 to 800 kDa (97). The KV motif of apo(a) is very similar to plasminogen, and it is followed by an inactive protease domain which shares 94% homology to plasminogen, at the C-terminus (96).

Lp(a) formation is believed to occur via a two step mechanism (98, 99). Firstly, the apo(a) glycoprotein forms a non-covalent interaction with apoB100 on the LDL particle, via low affinity Lys binding sites found in KIV repeat motifs 5-8 (Fig. 1.4a) (98, 99). In the second step the most C-terminal Cys4326 on apoB100, and a free Cys in KIV-repeat motif 9, are covalently linked via a disulfide bond (Fig. 1.4b,c) (97, 100). Apo(a) is known to be expressed exclusively by the liver (96). Most *in vitro* studies demonstrate that the assembly of the Lp(a) particle occurs on the extracellular surface of the hepatocyte (101, 102). Still, recent studies have also shown a potential for intracellular assembly, and Lp(a) (103) can form *in vitro* simply by mixing apo(a) in culture media with exogenous human LDL (104).

Plasma Lp(a) levels are primarily determined by genetics, and interestingly there is an inverse correlation between length of apo(a) and plasma concentrations of Lp(a) (96, 97). Individuals harboring apo(a) alleles with a low number of KIV type 2 repeats tend to have a higher concentration of Lp(a) than individuals expressing larger versions of apo(a) (96, 97). Up to 28% of the total molecular weight of the apo(a) protein can be accounted for by glycosylation (105). It is hypothesized that the increase in KIV repeats results in longer retention in the ER as the protein has to undergo increased N-glycosylation, protein folding, formation of more disulfide bridges, and interactions with ER resident chaperones (96, 97, 106). Because these larger isoforms are more complex, they are retained longer and therefore are susceptible to increased degradation. Smaller

isoforms are more efficiently produced, modified and secreted (96, 97). In addition to apo(a) length, regulation of mRNA levels by changes in the rate of transcription, or mRNA stability, also regulate the synthesis of this protein (96, 107). It is known that levels of apo(a) mRNA are downregulated in response to estrogen and testosterone (97, 108). Also, many potential methylation sites on the apo(a) DNA may serve to down regulate the expression of apo(a) (109). These distinct methods of regulating apo(a) production results in plasma concentrations of Lp(a) varying from 0.1 to 100mg/dL in various individuals (97).

Unfortunately, many limitations exist with regards to research on apo(a) and so little is understood about what the physiological role of Lp(a) is, how Lp(a) is formed, how it is catabolized, and whether or not Lp(a) plays a direct or synergistic role in the development of the atherosclerotic plaque (96). This is predominantly due to a lack of good animal models for the study of this particle. Apo(a) expression is limited to humans, non-human primates and hedgehogs (97). Overexpression of apo(a) in transgenic mouse models has also proven difficult since apo(a) can not form a covalent link with mouse apoB100 (110). All rodent forms of apoB100 lack the C-terminal Cys responsible for the disulfide link to apo(a) (96). In these transgenic mouse models, either human LDL had to be injected into the animals (110) or they had to be crossed with human apoB100 transgenic mice to have any Lp(a) formation (111-113). However, the studies performed on these Lp(a) transgenic mice presented conflicting results in showing Lp(a) having any atherogenic potential (112, 113).

It is important to note though that accumulation of Lp(a) seems to occur within atherosclerotic lesions only, and not in healthy vascular walls (97). It is hypothesized that apo(a) has a high affinity to binding fibrin, which is known to accumulate within the thickened intima (97). As described above, apo(a) is highly homologous to plasminogen which once proteolytically activated, functions to cleave fibrin (114). This hypothetical atherogenic potential of Lp(a) is also based on the observation that apo(a) accumulates only in the vessel walls of transgenic mice that also express fibrinogen (115). This affinity of Lp(a) for the intimal extracellular matrix, could potentially result in the additional accumulation of cholesterol rich particles within the lesion (97).

1.3.4 Gender: The risk for a cardiovascular disease event is significantly decreased in women compared to men prior to the age of 55 (116). Many studies have shown that this trend enjoyed by women prior to menopause is due to the atheroprotective effects of estrogen (116-118). Furthermore, many human studies have shown that postmenopausal women can significantly reduce their risk for developing atherosclerosis by taking hormone replacement therapy, again providing evidence that estrogen is beneficial for preventing atherosclerosis (117). The primary estrogen circulating in the plasma is 17β -estradiol and its protective actions are believed to be predominantly mediated by its two receptors; estrogen receptors α and β . These two receptors are expressed by two different genes, and there are some differences in tissue distribution but both are members of the steroid hormone receptor family of ligand activated transcription factors (118, 121).

Estradiol- 17β enters the cell by passive diffusion (118) and then binds to one of the estrogen receptors, which then either undergoes homo- or hetero-dimerization with another estrogen receptor molecule (122). This complex then moves into the nucleus where it mediates the transcription of target genes containing estrogen response elements in their promoters (118). Co-factors either inhibit or accelerate the transcription of these genes in response to the estrogen stimulus (118).

The predominant beneficiary effect of estrogen is on the ratios of LDL to HDL (116-118). With hormone replacement therapy, the levels of LDL cholesterol have been observed to go down while HDL goes up (123, 124). Both human and rat studies show that estrogen upregulates the expression of the LDLR (125, 126). Estrogen receptor mediated events also appear to affect the hepatic expression of a number of apolipoproteins including A, B, D and E (118, 127) as well as the serum concentrations of the atherogenic Lp(a) (118). Furthermore, studies have shown that estrogen decreases foam cell formation by protecting LDL from oxidation (128), and *in vitro* studies show that it inhibits uptake of modified LDL by activated macrophages (129).

The atheroprotective effects of estrogen are not limited to changes in plasma lipoproteins, which accounts for only 25-50% of the benefits of hormone replacement therapy (116). Estrogen receptor α is expressed in vascular and myocardial smooth muscle cells and endothelial cells (118). *In vitro*, estrogen has been shown to inhibit the

growth and migration of smooth muscle cells (130, 131). In contrast, estrogen has the opposite effect on endothelial cells by stimulating the production of endothelial growth factor (132, 133). This effect is especially beneficial in response to vascular endothelial injury where endothelial integrity is rapidly restored by the effects of estrogen (132). Estrogen administration has also been shown to affect other processes involved in atherosclerosis such as cytokine induced cell adhesion molecule expression (134), platelet adhesion (135), and macrophage lipid and cholesterol homeostasis (129, 136). Macrophages are less likely to be recruited to the atherosclerotic plaque with long term hormone replacement therapy since estrogen downregulates the expression of monocyte chemoattractant protein-1 (137) and vascular adhesion molecule (134).

The most predominant, non-lipid-altering effect of estrogen on the vascular wall is through a non-genomic process. Although the mechanism is not well understood, it is known that estrogen stimulates the rapid secretion of nitric oxide from the vascular endothelium without altering gene expression (118). Nitric oxide relaxes vascular smooth muscle cells and inhibits platelet activation (138). The increased availability of nitric oxide mediates increases in cyclic guanosine monophosphate, which opens the calcium activated K^+ channels in the vascular smooth muscle cells and promotes vasodilation (139, 140).

1.3.5 Homocysteine: Homocysteine (Hcy) is an sulfhydryl amino acid that is produced during the metabolism of Met (141). One of the primary functions of Met is to be converted to *S*-adenosylmethionine (AdoMet) which serves as a methyl donor for >100 methyltransferase reactions including DNA/RNA methylation, phospholipid synthesis, creatine formation, etc... (142). When AdoMet transfers its methylgroup to the acceptor substrate it is converted to *S*-adenosylhomocysteine (AdoHcy) (Fig. 1.5). AdoHcy is hydrolyzed to Hcy but this hydrolysis is reversible, highly favoring the production of AdoHcy (141). Hcy can be metabolized a number of ways. It can be remethylated back to Met by the actions of *N*-5-methyltetrahydrofolate methyltransferase (141). This reaction requires sufficient dietary intake of folate, is vitamin B₁₂ dependent and it takes place in all tissues (141). Betaine also serves as a methyl donor to convert

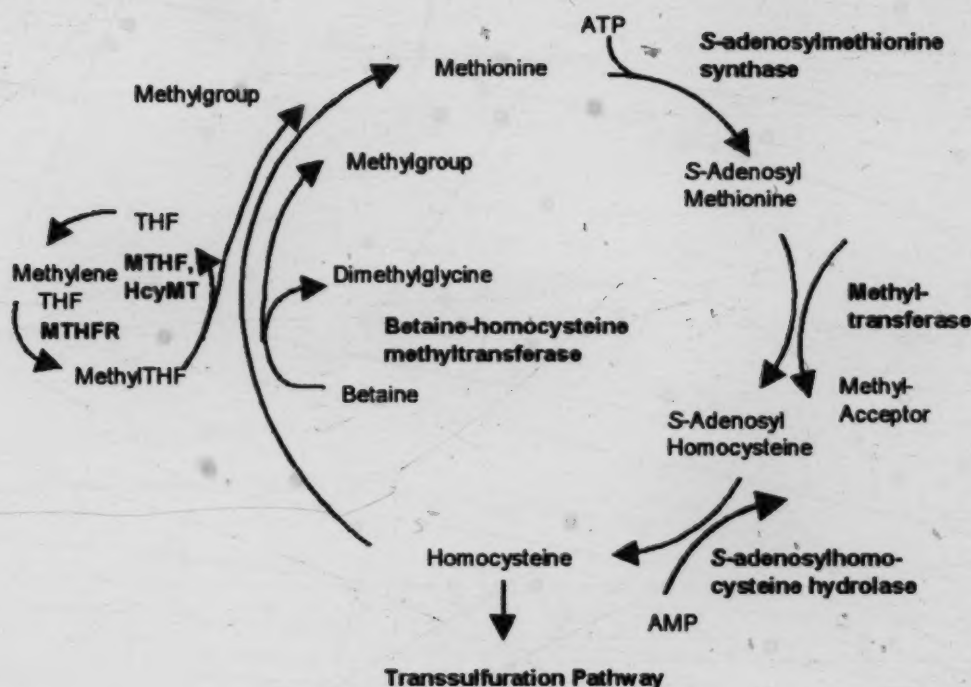


Figure 1.5: Homocysteine Metabolism

Methionine is an essential amino acid used primarily in protein synthesis. In addition, Met can be converted to AdoMet, which serves as a methyl donor for many methyltransferase reactions. When AdoHcy builds to excess, it is hydrolyzed to Hcy, a known detrimental amino acid. Hcy can be remethylated to Met by one of two pathways. Methyltetrahydrofolate (MethylTHF) donates a methylgroup to Hcy via methyltetrahydrofolate, homocysteine methyltransferase (MTHF, HcyMT). Tetrahydrofolate (THF) can be converted back to MethylTHF by first converting to methylene tetrahydrofolate (MethyleneTHF) and this step requires pyridoxal 5' phosphate. MethyleneTHF is then reduced to MethylTHF by Methylenetetrahydrofolate reductase (MTHFR). Mutations in MTHFR are common in the population and depending on the severity may result in mildly elevated plasma Hcy levels, or homocystinuremia. In the liver, oxidized choline (known as betaine) also can act as a methyl donor to Hcy. When Met levels are sufficient, Hcy is shunted into the transsulfuration pathway for eventual excretion (figure adapted from reference 141)

Hcy to Met, however this reaction is limited to the liver (141). In cases where the AdoMet concentration is sufficiently high, Hcy is catabolized via the transsulfuration pathway (141). Here, cystathionine β -synthase (CBS) irreversibly condenses Hcy with serine to form cystathionine, which is later hydrolyzed into Cys and α -ketobutyrate. The excess Cys is either oxidized or is excreted in the urine. The first two steps of the Hcy catabolic pathway are vitamin B₆ dependent (141).

The first observation that increased total plasma Hcy could be a cardiovascular disease risk factor was in 1969 when McCully observed that a number of homocystinuric patients developed early onset atherosclerotic disease (143). Homocystinuremia is a condition where patients have extremely high levels of plasma Hcy due to a genetic error in either the tetrahydrofolate methylation pathway, or in the transsulfuration pathway (Fig. 1.5) (141). In 1976, Wilcken and Wilcken observed that patients with cardiovascular disease frequently had elevated levels of plasma Hcy (144). Since these initial observations, many studies have been performed examining plasma Hcy concentrations and cardiovascular disease and they suggest that an elevated Hcy concentration is an independent risk factor for this disorder (145-147).

The physiological role of Hcy, other than a precursor of Met, is unknown (142) and most studies have shown Hcy to only have deleterious effects. Hcy can undergo auto-oxidation and the reactive oxygen species formed during this process may contribute to vascular endothelial cell injury (147). Both animal and human studies have shown that high Hcy levels induced either by a direct Hcy infusion, or an oral Met load, had deleterious effects on endothelial cells (148, 149). *In vitro* studies have implicated Hcy with stimulating smooth muscle cell proliferation (150), promoting LDL oxidation (151), inducing collagen synthesis by vascular smooth muscle cells (152), stimulating tissue inhibitor of metalloproteinases-1 (153), and promoting Lp(a) binding to fibrin (154). Furthermore, endothelial damage as a result of increased plasma Hcy has been shown *in vivo* to cause a decrease in nitric oxide mediated vasodilation (155). All these effects suggest that Hcy contributes to the advancement of the atherosclerotic lesion.

Most recently, high intracellular Hcy levels have been shown to induce an ER stress response in a number of human derived cell lines (156, 157). In the human hepatocyte derived HepG2 cell line, the Hcy induced ER stress was shown to stimulate

the expression of SREBP-1 (157). This resulted in an increase in expression of a number of enzymes involved in the synthesis of cholesterol. CBS^{+/-} mice were fed a diet to induce hyperhomocysteinemia. In the livers of these animals, there was an increased steady state expression of SREBP-1 and LDLR. Increased synthesis and uptake of TG and cholesterol into the livers resulted in hepatic steatosis, and there was an increased rate of lipid secretion into the plasma. These results implicate Hcy in regulating lipid metabolism, and therefore LDL/HDL ratios in the plasma (157).

Although Hcy seems to induce all of these deleterious effects, it has still not been established that elevated plasma Hcy contributes to increased risk for cardiovascular disease in otherwise healthy individuals (146). A common polymorphism in the methylenetetrahydrofolate reductase (Fig. 1.5) gene results in hyperhomocysteinemia. However, individuals harboring this polymorphism are at no greater risk for developing cardiovascular disease unless they are already predisposed to the condition (146). Although CBS⁺ (158) and methylene tetrahydrofolate reductase deficient mice (142) develop severe homocystinuria, there no obvious development of atherosclerosis in these animals unless they reach old age (142). Recent human studies suggest that moderate homocysteinemia does not induce atherosclerosis on its own but it enhances the risk in people already diagnosed with cardiovascular disease (146). Interestingly, a study from Norway showed that elevated plasma Hcy was the strongest predictor for mortality in people already diagnosed with cardiovascular disease; above plasma cholesterol concentrations, Lp(a) and hypertension (159). Almost 5 years after the initial plasma Hcy readings, only 4% of patients with an initial plasma Hcy concentration of <9 $\mu\text{mol/l}$ had died compared to 27% of patients with a concentration of $\geq 20 \mu\text{mol/l}$ (159).

1.4 Synthesis and Secretion of VLDL Particles

Since high LDL/HDL ratios correlate with an increased risk for cardiovascular disease, a great deal of effort has been invested in understanding the synthesis and secretion of the LDL precursor, VLDL. VLDL is predominantly produced in the liver. However, recent studies have shown that the rodent yolk sac (160), chick kidney (161) and the murine and human heart (162) are also capable of synthesizing and secreting

apoB100 containing lipoproteins. In the yolk sac, the endodermal cells express a number of lipoprotein receptors such as megalin, cubulin, SR-B1 and the LDL receptor related protein (163). These receptors take up lipoproteins from the maternal pools and the lipids are then packaged into VLDL like particles that are transported to the developing embryo (10, 163). Rodent embryos are extremely dependent on this process since apoB null mice die *in utero* (164).

With regards to the heart, it is known to express all the proteins required for VLDL synthesis and secretion (162). Compared to the liver, the rate of apoB100 secretion from the heart is very low (162). The heart is not a major TG synthesizing organ and it mainly oxidizes fatty acids for energy use. Perhaps, the ability to secrete lipoproteins is a protective mechanism for times when free fatty acids have accumulated to toxic levels such as post-ischemia (162). The chick kidney is capable of secreting apoB at the liver equivalent of 15%, but this capability is not observed in mammalian species (161).

Because the liver is the major VLDL secreting organ, almost all studies have been performed in either rodent primary hepatocytes or hepatoma derived cell lines such as human HepG2 and rat McArdle RH7777 cells. From these studies it has been determined that apoB100 is translated constitutively and the rate of apoB secretion is primarily dependent on the availability of lipids (54). Less is known about the transcriptional control of apoB, and studies have been limited to understanding the regulation of its tissue distribution (165). The following is a summary of what is known about the apoB protein, and the requirements for its secretion.

1.4.1 ApoB100 Protein Structure: ApoB100 is a 4536 amino acid protein with a molecular mass of 513kDa (9). The highly hydrophobic nature of the apoB protein distinguishes it from all other apolipoproteins because apoB is not exchangeable (9, 54). The amino acid sequence suggests a-pentapartite structure with alternating α helices and β sheets (NH_2 - α_1 - β_1 - α_2 - β_2 - α_3 -COOH) (Fig. 1.6) (166). The first 27 N-terminal amino acids target the apoB protein to the ER, and this signal sequence is cleaved prior to secretion (9). The first 17% of apoB (apoB17) makes up the α_1 region of the protein (167). This region is extremely globular and 6 of the total 8 disulfide bridges found in

apoB are clustered in this region (168, 169). ApoB17 has a low affinity for lipids compared to the rest of the protein sequence and when overexpressed in hepatoma cell lines it can be secreted in the absence of lipids (170). This region is however, essential for the proper association of the apoB100 protein with the lipid particle. When proper folding of the α_1 region is inhibited, the apoB protein is unable to assemble with lipid (168). The role of this region is unknown but when expressed on its own it is able to associate with phospholipids, suggesting it recruits the phospholipid monolayer of the lipoprotein particle (171). As well, apoB17 has been shown to associate with another important protein in VLDL assembly-MTP (172), which will be described in section 1.4.3C.

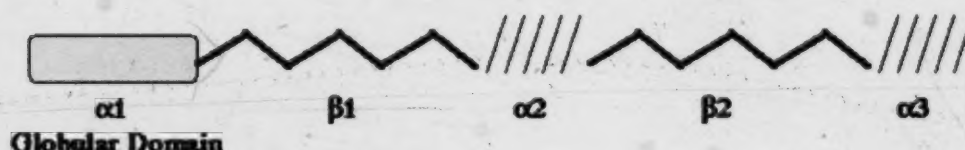


Figure 1.6: Structure of ApoB100

The hypothetical secondary structure of apoB100 is pentapartite based on computer analysis of the amino acid sequence (166). The first 17-20% of apoB is considered the first α helical region and it is highly globular (166). Two β -sheet regions are located approximately between residues 827-1961 and 2611-3867 (166). These regions are believed to give apoB its highly hydrophobic, non-exchangeable properties (54). The 2 additional α -helical regions are predicted at residues 2103-2560 and 4061-4338 (166). Some of these α -helical regions resemble those found in exchangeable apolipoproteins and are believed to give apoB flexibility so that it is able to respond to large changes with regards to lipid particle size (166) (figure adapted from reference 250).

The other two α -helical regions are clustered in the regions of residues 2103-2560 and 4061-4338 (166). The α -helices that compose these two regions are not long enough to be considered transmembrane yet they are amphipathic and so it is hypothesized that they lay on the particle with their non-polar sides facing the lipid (9). The secondary structure of all the exchangeable lipoproteins consists predominantly of amphipathic α -

helices (9, 166). Therefore, it is believed that these α -helical regions confer reversible lipid binding, perhaps so that the protein can adjust to required changes in its structure as the particle size decreases with lipolysis (166).

The anti-parallel, amphipathic β -sheet regions of the apoB protein promote strong, irreversible lipid binding (54). The non-polar faces of these β -sheets are believed to penetrate deep into the phospholipid monolayer of the lipoprotein (54). The β -sheets are also hypothesized to have an additional role in the ER lumen prior to secretion on the lipoprotein particle. Proteolytic cleavage studies on microsomal fractions of rat liver (173), primary hepatocytes (174) as well as HepG2 cells (175) suggest that in the absence of sufficient lipid apoB exists as a transmembrane protein in the ER. As described above, the α -helices found in the apoB protein are not long enough to traverse a bilayer. Yet, the β -sheet structures found between apoB21 and B41 share 43% sequence identity with those of porins (9, 176). In porin, these β -sheet structures form oligomeric structures that span the membrane bilayer (177). Therefore, it is hypothesized that the β -sheet structures allow the transmembrane conformation of apoB in the ER membrane when levels of core lipids are limited (9, 54).

Beyond the unique size and secondary structure of apoB, there are many post-translational modifications to this protein. As already discussed, there are 8 disulfide bridges throughout the length of the protein and they are essential for the proper folding and assembly with lipid (167). In addition, there are 20 potential N-glycosylation sites throughout the apoB100 sequence (178). Studies in HepG2 cells where apoB₁₀₀ glycosylation was inhibited with tunicamycin suggest that glycosylation is not required for the secretion of apoB100 containing lipoproteins (179, 180). Still, the secretion of apoB100 in all of these studies was decreased due to increased degradation, suggesting glycosylation may play a role in the structure of apoB (179, 180). Palmitoylation of apoB is necessary for the proper intracellular sorting of apoB into the secretory pathway (181). Finally, apoB100 has been shown to be phosphorylated, but it is not known which residues are phosphorylated and what regulatory role this plays (182, 183).

Overall, it is hypothesized that the apoB100 protein covers between a third and half of the LDL particle surface (178). Electron microscopy of LDL particles with monoclonal antibodies bound to specific epitopes on the apoB100 protein has resulted in

a "ribbon and bow" model for the conformation of the protein (184, 185). These studies suggest that the first 89% of the apoB sequence forms a thick ribbon that surrounds the circumference of the LDL particle (185) (Fig. 1.7). The last C-terminal 11% folds back and crosses the ribbon and this structure resembles a bow (185). When the particle is VLDL sized, it is hypothesized that the bow crosses over the LDLR binding region of the apoB100 protein at residues 3359-3369 (185, 55). As the VLDL particle shrinks to LDL size, apoB100 undergoes considerable structural changes such that the LDLR binding region is exposed and the particle can now serve as a ligand (55).

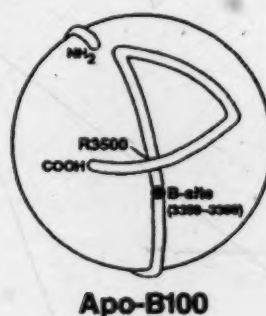


Figure 1.7: Ribbon and Bow Model for ApoB100 Association with Lipid Particle

The first 89% of the apoB100 protein circles the lipid particle, while the C-terminal 11% folds back over the ribbon forming a bow. The bow portion is proposed to modulate the affinity of the apoB containing particle to the LDLR. When the particle is VLDL sized, the bow crosses over the hypothesized LDLR binding region (B-site). As the particle undergoes lipolysis, apoB100 undergoes structural changes that allow the bow to move away from B-site exposing the LDLR binding region (55). Mutagenesis studies suggest that at this point, Arg3500 interacts with the C-terminal tail, keeping it away from the LDLR binding region (figure taken from reference 55)

1.4.2. ApoB48: In the intestine, the 241 kDa apoB48, which constitutes the N-terminal 48% of apoB100, is secreted on CM particles (9, 54, 178). In a unique post-transcriptional editing step on the apoB100 mRNA, cytidine 6666 is converted to a uridine, resulting in a premature stop codon (186). This is catalyzed by a multi-unit complex, of which APOBEC-1 is the known catalytic cytidine deaminase (187). In

humans, apoB48 is produced only in the intestine. However in rodents, this editing process occurs in the liver as well (9, 54). This is important with regards to apoB100 research since the most commonly used models are rat and mouse livers, primary hepatocytes and hepatoma derived cell lines. Although much has been learned from these model systems, many of these studies have shown differences between the hepatic secretion of apoB48 and apoB100. In all cases, apoB48 can be secreted on lipoprotein particles that range in size from HDL to VLDL (188, 189). In contrast, apoB100 can only be secreted on VLDL sized particles demonstrating that the lipid requirements for apoB100 secretion are much higher than for apoB48 (188). Processing of apoB48 compared to apoB100 also differ in response to treatment (190). Treatment of McArdle RH7777 cells (hepatoma derived cell line from rat) with Brefeldin A resulted in continued secretion of apoB48 on HDL sized particles, whereas B48 containing VLDL particles were not made (190). ApoB100 could not be located in the microsomal lumen of these cells, but was associated with the membrane as a transmembrane protein (190). These studies suggest that the synthetic pathways for an apoB48 containing lipoprotein may differ versus an apoB100 containing lipoprotein (190).

1.4.3. Requirements for VLDL Secretion:

A) ApoB Protein Length: As outlined above, the apoB protein is required for the secretion of the VLDL particle. It is important to note that the long lengths of both apoB48 and B100 are essential for the production of the large CM and VLDL particles (9). Many studies have shown an inverse correlation between the length of the apoB protein, and the size of the particle it is associated with (191, 192). For example, expression of C-terminally truncated versions of apoB in McArdle RH7777 cells demonstrated that the N-terminal 37% of the apoB protein is required for assembly into a VLDL particle (193). Smaller versions of apoB are only able to form LDL or HDL sized particles (19). Although apoB37 is able to form VLDL particles in McArdle cells, the development of a mouse expressing only apoB38.9 demonstrated otherwise. Even though secretion of apoB38.9 was very efficient, these animals developed fatty livers due to a reduced ability to export TG (194). It is hypothesized that the apoB protein needs to be of sufficient length that it surrounds the circumference of the particle, therefore

providing structural integrity (9). ApoB length can be a problem with patients suffering from familial hypobetalipoproteinemia due to a mutation in the apoB gene (9, 194). Truncated forms of apoB ranging from apoB2 to B89 have been identified in these patients (194). These patients have low plasma cholesterol and apoB, and they may develop fatty livers (194).

B) Triglyceride: Triglycerides comprise the majority of the neutral lipid core of VLDL particles and in this way are required for VLDL secretion. Because apoB100 is so hydrophobic, it requires the presence of sufficient amounts of lipid for its assembly into a particle (195). In the absence of sufficient lipid, apoB100 is eventually degraded (195). This is particularly obvious in the McArdle RH7777 and HepG2 cell lines where VLDL secretion is dependent on the treatment of these cells with oleate (188, 196, 197). Oleate stimulates the production of TG, prevents the degradation of apoB and increases the production and secretion of VLDL sized particles (198). If the oleate treatment is removed, these cell lines can only maintain VLDL secretion for a short time and then lipoprotein secretion returns to normal (199, 201). Normally, both of these hepatoma cell lines are unable to produce VLDL and only secrete LDL/HDL sized particles (188, 197). Unlike hepatoma derived cell lines, primary hepatocytes do not rely on the presence of oleate for significant VLDL secretion. Studies indicated that treatment of hepatocytes with oleate did not increase the secretion of apoB but merely increased the size and buoyancy of the particles. These cells also maintained VLDL secretion after the oleate treatment was removed (189).

From the above studies it is now understood that the ability of a cell to mobilize TG stores is also an important requirement for VLDL secretion (200). The majority of cellular TG stores are localized in the cytosol, and only minimal quantities are stored in the ER lumen (200, 201) (Fig. 1.8). The TG found in the cytosolic pool has been shown to contribute to the VLDL secretory pool via a lipolysis step to diacylglycerol (DAG)/MAG followed by re-esterification to TG. Through this process, the TG crosses the ER membrane to the VLDL secretory pool (200). Two groups have shown that 60-70% of VLDL derived TG is via a lipolysis/re-esterification process (202, 203). Both hepatoma cell lines have lost the ability to mobilize the cytosolic stores and transfer them

to the ER, which explains their reliance on oleate treatment for VLDL secretion (199, 201). In the presence of oleate, the smaller ER luminal TG pool is maintained and not depleted and so there is sufficient accessible TG for VLDL particle assembly (201).

Two candidates that are hypothesized to play roles in mobilizing TG stores are triacylglycerol hydrolase (TGH) and arylacetamide deacetylase (AADA) (Fig. 1.8). While TGH is loosely associated with the luminal side of the ER membrane, AADA is an ER transmembrane protein (200). TGH is a 60kDa member of the carboxylesterase family of proteins, and has been shown, *in vitro*, to have lipolytic activity (204-206). Tissue expression of TGH is limited to liver, intestine, heart, kidney and adipose tissue (206). McArdle and HepG2 cells do not express TGH, but overexpression of TGH in McArdle cells increased apoB and TG secretion significantly (199). Furthermore, TGH is associated with lipid droplets in the liver and immunocytochemical studies localized TGH to the liver cells surrounding capillaries (205). Finally, TGH is inhibited by known lipase inhibitors (205). AADA is a homologue of hormone sensitive lipase and expression is limited to liver, intestine and adrenal gland (200). AADA is not expressed in HepG2 cells but overexpression resulted in a 2-3 fold increase in TG secretion (200). Finally, both enzymes are not expressed until weaning, when VLDL secretion becomes significant (200). The accumulation of the above evidence suggests that either TGH, or AADA, could play a significant role in the mobilization of TG stores (200). Diacylglycerol acyltransferase is the enzyme responsible for the reacylation of the hydrolysis products to TG (200).

Another contributor of TG to the VLDL particle is phospholipid hydrolysis (207). Overproduction of PC does not result in an accumulation of PC mass but an increase in turnover (208). In studies where PC synthesis was blocked, it was demonstrated that the DAG produced after PC hydrolysis is shunted to TG synthesis (209, 210). Finally, it was shown recently that a major TG biosynthetic pathway in yeast utilizes fatty acids from phospholipid hydrolysis (211). The advantage of phospholipid mobilization to TG is that the lipids are already within the membrane, whereas the hydrophobicity of TG makes it difficult to insert into the membrane for access by membrane resident or luminal hydrolases.

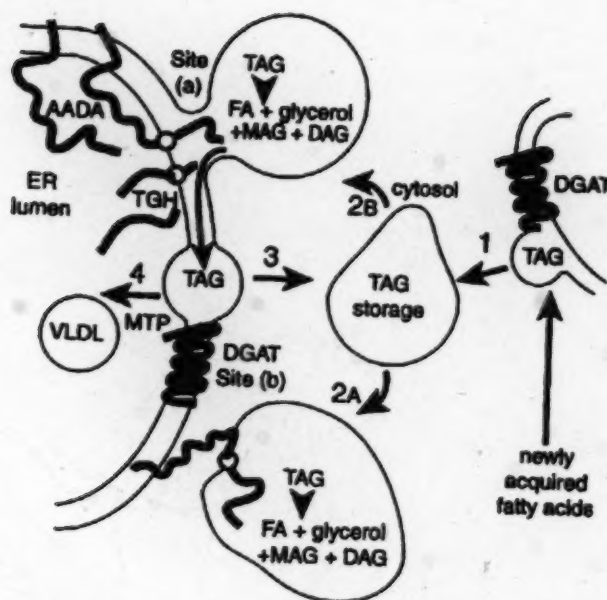


Figure 1.8: Mobilization of Intracellular TG for VLDL Secretion

1. Newly acquired free fatty acids in the liver are synthesized into TG by the activity of diacylglycerol acyltransferase (DGAT), localized at the ER. This TG (TAG) is then stored in the cytosol, in a phospholipid bound vesicle. 2. The storage vesicle needs to come into close proximity with the ER. 2A. One possibility is that a cytosolic facing, ER enzyme is responsible for the hydrolysis step. In this case the vesicle needs to be very close to the ER. 2B. Another possibility is a fusion step with the outer layer of the ER bilayer. Here, either TGH or AADA could potentially access the TG and hydrolyze it to free fatty acids (FA), MAG, DAG and glycerol. DGAT re-esterifies the products to TG. 3. The newly re-synthesized TG can either be recycled back to the cytosolic TAG storage pools or 4. transferred to pools accessible for assembly into VLDL particles (figure taken from reference 200).

C) Microsomal Transfer Protein: MTP is a 97 kDa protein that resides in the ER lumen via its non-covalent interaction with protein disulfide isomerase (PDI) (212). *In vitro*, MTP mediates the transfer of lipids between liposomes with lipid transfer rates decreasing in order of TG > CE > DAG > cholesterol > PC (213). All studies of MTP confirm its activity is necessary for apoB and VLDL secretion (9, 54, 212). Firstly, the essential role of MTP for apoB containing particle assembly is strongly demonstrated in abetalipoproteinemic patients. These people do not have a functional MTP protein and are unable to assemble CM or VLDL particles resulting in inefficient uptake and transport of dietary lipids and lipid soluble vitamins (214). Secondly, co-expression of apoB with MTP in COS-1 (215) and COS-7 (216) cells alone was sufficient to make these cells capable of secreting lipoproteins (216). Finally, many animal and cell culture studies have been performed using a variety of MTP inhibitors. The consensus of all these studies is that inhibition of MTP activity results in decreased CM and VLDL secretion (217-220).

Despite all the work, the specific role of MTP with regards to the VLDL assembly process is not yet clear. It is well established that MTP physically interacts with apoB (172, 221-224) and this interaction is necessary for the assembly of the VLDL particle (223). However, whether or not MTP activity is required for the complete translocation of apoB48 and B100 into the ER lumen is controversial (225-228). With regards to what stage of VLDL assembly MTP is required, different groups have obtained conflicting results (229, 230). It does appear that MTP plays a role in maintaining the ER lipid storage pool (230-232). Although MTP is not responsible for mobilizing TG (231), it may play a role in transferring newly reacylated TG from the ER membrane to the storage pools which are accessible for assembly into VLDL (230-232).

Development of the MTP knockout mouse resulted in embryonic lethality, since MTP is essential for lipoprotein assembly in the yolk sac (233). Then 2 separate research labs created liver specific, conditional MTP knockout mice (234, 235). Raabe *et al.* (234) observed a complete loss of apoB100 in the plasma of these conditional knockouts, but only a 20% reduction in total plasma apoB48. It is important to note that only total apoB was analyzed and not the buoyant densities of the apoB48 particles secreted. As

described above, apoB48 secreted from liver can assemble into particles ranging from HDL to VLDL so it is unknown whether or not loss of MTP affected this aspect of apoB48 secretion (234). Chang *et al.* (235) also observed a complete loss of plasma apoB100 and a much larger reduction in total plasma apoB48 than observed by the other group. Both groups observed significant decreases in plasma VLDL cholesterol and TG (234, 235) and Chang *et al.* (235) also demonstrated a specific reduction in TG secretion from their conditional MTP knockouts. Raabe *et al.* (234) performed electron microscopy on the livers of their conditional MTP knockouts and observed a complete loss of ER and Golgi lipid stores. This result confirmed that MTP plays a role in maintenance of the luminal ER lipid stores that are available for assembly into VLDL (234). Nevertheless, even with the development of these mice, not all the questions surrounding the role of MTP have yet been answered.

D) Phosphatidylcholine: In humans, PC constitutes approximately 60% of the total phospholipids that make up the outer monolayer of all lipoproteins (236). In mammals, the Kennedy pathway synthesizes PC in all nucleated cells, and it relies on the dietary intake of choline (237). The secondary Phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway is located specifically in the liver where it is responsible for 20-40% of the total PC made in this organ (238). The PEMT pathway uses phosphatidylethanolamine (PE) and AdoMet as substrates (237). This pathway is a very important source of *de novo* synthesized choline and PC when choline is absent from the diet (239).

To examine the role of PC in lipoprotein secretion Yao and Vance used hepatocytes isolated from rats fed a choline deficient (CD) diet for 3 days. The hepatocytes isolated from the CD animals had a 6-fold accumulation of TG compared to control hepatocytes (240). Additional analysis confirmed that plasma VLDL levels were decreased in these animals compared to the control fed rats (241). These results suggested that VLDL secretion was already impaired as a result of inhibiting the primary pathway of PC synthesis. These hepatocytes were then cultured either in the presence or absence of choline and/or Met (240). From these studies it was determined that apoB, but not apoA1 secretion, was greatly inhibited when PC biosynthesis was completely

impaired (240). However, if either of the two pathways were intact with choline or Met supplementation, VLDL secretion continued (240). Later studies performed by this same group confirmed that the PC headgroup was specifically required for VLDL secretion and other phospholipids could not compensate (242). More recently, the requirement of PC for VLDL secretion was confirmed in the PEMT knockout (*Pemt*^{-/-}) mouse fed a CD diet (239). These animals died after 3 days because they were unable to produce PC. Lipoprotein analysis of plasma confirmed that all lipoprotein fractions were essentially absent from these animals, validating the important role of PC in lipoprotein metabolism (239).

E) ApoB Translocation and Folding: Assembly of the VLDL particle occurs within the lumen of the ER and so it is necessary for apoB100 to be co-translationally translocated into the ER lumen (9, 54). As described above, the structure of apoB100 is extremely large and complex and so there are a number of requirements for its proper translocation and folding in the ER. Firstly, apoB translocation is facilitated by the presence of adequate lipid. Throughout the apoB100 sequence there are potentially 40 pause-transfer sequences where translocation through the Sec61 complex is temporarily arrested (9, 54). One hypothesis suggests these temporary pauses allow time for the translocated portions of apoB to fold properly and associate with either lipid or the ER membrane (54). Interestingly, studies have shown that ER membrane composition may impair restarting of translocation. VLDL secretion is severely inhibited when the rat liver membranes are enriched in phosphatidylmonomethylethanolamine (PMME) (243). Using cell free translation and translocation systems, it was shown that translocation of paused apoB15 was unable to restart in PMME enriched microsomes (244). Although PMME only contributes 0.1% of the total phospholipid under physiological conditions, these studies suggest that the proper lipid environment in the ER membrane is required for apoB translocation (244).

In addition, apoB100 has been shown to interact with a number of ER resident chaperones including calnexin (245), ER60/ER57 (246), BiP/GRP78 (247), GRP94 and calreticulin (247) and it is believed these chaperones facilitate the proper translocation and folding of apoB. For example, inhibition of the interaction of apoB with calnexin

resulted in increased apoB degradation (245). BiP is hypothesized to interact with hydrophobic domains of unfolded proteins, and is also believed to facilitate translocation into the ER (247). The exact role of GRP94 and apoB100 folding is unknown, however studies suggest this chaperone interacts in the later stages of folding and assembly with lipid (247). Proper folding is also facilitated by free PDI, which is hypothesized to play a role in the disulfide bond formation within the apoB protein (248). Overall, little is understood concerning the specific roles each chaperone plays, however it is likely that the activities of these chaperones facilitate proper assembly of apoB with the lipid particle (249).

1.4.4 Theories on the Mechanism of VLDL Assembly: Over the years a number of hypotheses have been proposed to explain the specific mechanism of assembly of apoB with the lipid particle (54). Presently, the most popular theory is the two step model (Fig. 1.9A) (250, 251). Here, as the apoB is translocated into the rough ER lumen it associates with phospholipid and a small amount of neutral lipid (188, 249). MTP activity has been shown to play an essential role in the transfer of lipid to apoB during this first step of VLDL particle formation (229). After the translocation of apoB is complete, it is associated with a small primordial particle, which then moves to the junction between the rough and smooth ER (250, 252). By an unknown mechanism, the bulk neutral lipid is added at a second step (188, 249). One theory is that neutral lipid droplets localized in the smooth ER fuse with the HDL sized particle (253). The evidence suggests that MTP is not involved in the second step of VLDL production (229). The two-step model proposed above is based on the observation that small apoB48 and B100 HDL sized particles can be observed within the microsomal lumen, post sodium carbonate extraction (188, 249). However, at least in the case of apoB100, these small particles must mature to VLDL size before secreted, providing the basis of the two-step model.

The second model proposed for VLDL assembly is the one step model (Fig. 1.9B). Here, the bulk of the neutral lipid core is added either co-translationally, or very early on in particle assembly (54). This model is based on the observation that full sized VLDL particles have been isolated in the rough ER (254). This observation has however

been refuted by other groups (255). In addition one group proposed apoB48 and apoB100 containing lipoproteins utilized different mechanisms (256). In their studies they showed that apoB48 VLDL was produced via the two-step model, whereas bulk TG was added to apoB100 co-translationally in one step (256). Later on, this same group opposed its own work by demonstrating bulk lipid addition to apoB100 containing lipoproteins occurred later in particle assembly (230). In their studies they demonstrated a requirement for MTP activity in this second step (230).

The third and least popular model with regards to VLDL assembly is the Golgi model where bulk neutral lipid is added in the Golgi (Fig. 1.9C) (54, 257, 258). This model has recently enjoyed a resurrection in evidence. Firstly, a group recently isolated Golgi from rat liver and showed that apoB100 sized particles varied in size from 35 nm to 60nm, even though only the large particles are secreted (259). From this study it was concluded that with regards to the two-step model, the bulk lipid addition is in the Golgi. Also, by an unknown mechanism, Golgi expression of phospholipid transfer protein has also been shown to be required for the secretion of apoB100 containing lipoproteins (260). Finally, Yao *et al.* recently presented work where they showed apoB100 remained associated with membrane until the Golgi fraction, where bulk lipid addition occurred (261).

Finally, the main proponents of the two-step model recently showed that bulk VLDL lipid accumulation was an ADP-ribosylation factor 1 and phospholipase D dependent process (262). Evidence strongly suggests these two enzymes are required for COP-1 vesicle budding (263). COP-1 vesicles transport components within the Golgi complex, as well they regulate retrograde transport from the Golgi back to the ER, and some COP-1 vesicles have been observed to bud from the ER itself (264). The authors propose, amongst a number of hypotheses, that perhaps bulk VLDL TG is added during the budding of a COP-1 vesicle which would be an all new model (262). What is obvious from this is that although an incredible amount of work has been devoted into examining the synthesis of the VLDL particle, the mechanism, and all the proteins involved have not yet been clearly elucidated.

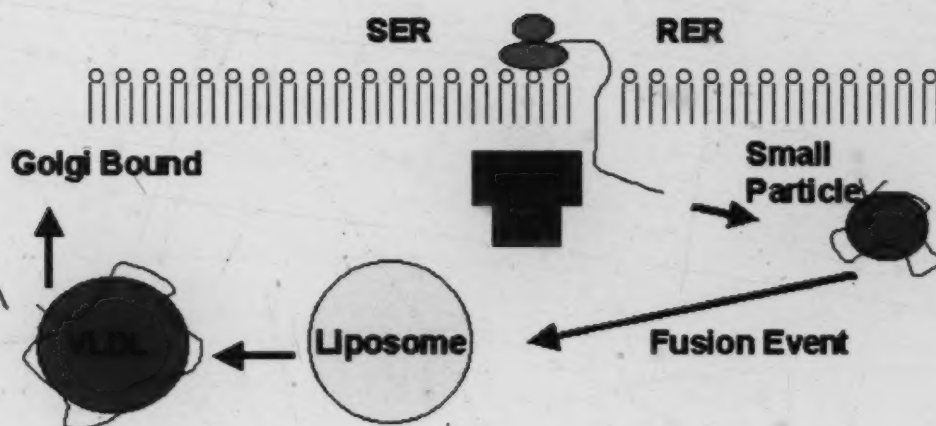
Figure 1.9: Hypotheses Concerning the Mechanism of VLDL Particle Formation

A. Two Step Model. As the apoB protein is co-translationally translocated into the lumen of the rough ER (RER), it associates with a small amount of lipid to form an HDL sized particle. MTP is responsible for transferring lipid to the apoB protein at this stage of particle synthesis. The primordial particle then moves towards the smooth ER (SER) where the bulk of the neutral lipid core is added by an unknown mechanism-perhaps via a fusion step. This second step requires ADP-ribosylation factor 1 and phospholipase D activities. The VLDL sized particle is now ready to continue through the secretory pathway.

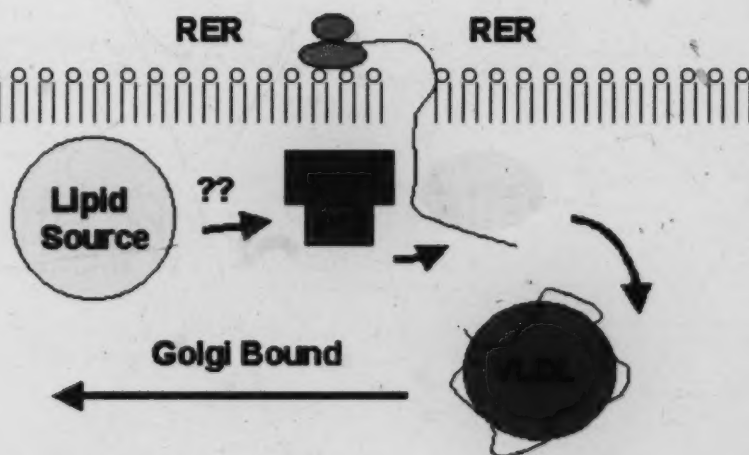
B. One Step Model. The bulk of the neutral lipid core is added to the apoB protein as it is co-translationally translocated into the ER lumen. The mechanism for this lipid transfer has not been elucidated, however one possibility is MTP. After the VLDL particle is synthesized it goes through the secretory pathway.

C. Particle Formation in the Golgi. As in the 2-step model, apoB associates with a small amount of lipid to form an HDL sized particle. This small particle then is transported to the Golgi where by an unknown mechanism, the bulk neutral lipid core is added. The VLDL particle then continues through the secretory pathway.

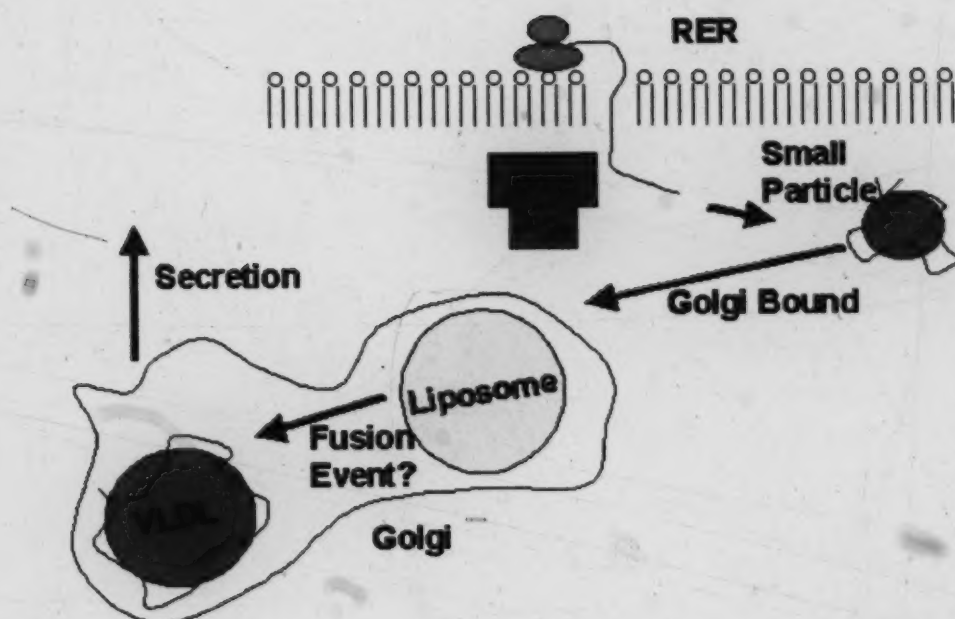
A.



B.



c.



1.5 Introduction to Phosphatidylcholine Metabolism:

PC is the primary phospholipid in eukaryotes, where it plays many roles. Firstly, PC comprises 25-50% of the total phospholipid content of eukaryotic bilayer membranes (265). The total PC concentration varies depending on the species of animal, tissue, and cellular organelle. (265, 266). In this way PC plays an extremely important role in the maintenance of membrane structure, and therefore cellular and organelle integrity. PC necessity is shown during cell division where active PC synthesis is required to provide the extra membrane structures needed for the development of the two daughter cells (267). However, the requirement for PC is most avidly demonstrated by a temperature sensitive mutant Chinese hamster ovary (CHO) cell line, where the cells are unable to synthesize PC at the restrictive temperature and eventually succumb to apoptosis (268, 269).

PC is also a precursor to a number of second messengers within signal transduction pathways. Release of arachidonate from the *sn*-2 position of PC by the actions of phospholipase A₂ serves as an important precursor of eicosanoids (13). LPC produced by this same reaction is also a bioactive molecule (37). On the other hand, degradation of PC by phospholipase C generates DAG, an important activator of protein kinase C, a key regulator of many cellular functions (13). PC can also donate its phosphoryl headgroup to ceramide to produce sphingomyelin (SM) and DAG (270). Increased levels of ceramide can stimulate the apoptotic cascade (41), whereas SM is an important component of lipid rafts which are known to play an essential role in a number of signal transduction pathways (271). Phospholipase D removes the choline headgroup of PC to produce phosphatidic acid, which can later be hydrolyzed also to DAG (13). Most recently, the conversion of PC to phosphatidic acid and DAG has also been shown crucial in vesicle budding in the cellular secretory pathway (263).

In higher eukaryotic systems PC plays other important roles. As described in section 1.4.5, active PC synthesis in the liver is required for VLDL secretion. In CD conditions VLDL particles and apoB100 are degraded in a post-ER compartment (272). This requirement for PC is because it is the most predominant phospholipid of the outer, soluble monolayer that surrounds the neutral core. PC is the main phospholipid for all

the lipoprotein groups (236). In the case of HDL, not only is PC the main phospholipid of the soluble outer membrane, but also it is required for the development of the CE rich core (8, 58). LCAT removes a fatty acyl group from PC and transfers it to the free cholesterol found also on the outer monolayer to produce CE (8, 58). As well, the other two major phospholipids found in the plasma are LPC and SM, both of which are derived from PC (273).

Additional demands on liver PC levels are by way of bile secretion. PC makes up >95% of the phospholipid in bile where it plays a crucial role in producing micelles with bile acids (274). Cholesterol secretion into bile is directly linked with, and is dependent on PC secretion (274). The multi-drug resistance protein 2 is a PC flippase expressed on the canalicular surface of hepatocytes, and it is hypothesized to translocate PC from the hepatocyte into the bile (275). In mice where this gene has been knocked out, there is a complete absence of PC in the bile and these animals endure extensive liver damage (276).

Finally, PC is a fundamental component of lung surfactant, a lipid/protein containing mixture that prevents alveolar collapse upon exhaling (277, 278). Babies born prematurely sometimes suffer respiratory distress syndrome because they are at a developmental stage where lung surfactant is not yet efficiently produced (278). The PC in lung surfactant is unique in that both fatty acyl species are palmitate (16:0) (277, 278). How the lung produces such a large quantity of this unusual PC species is not yet known.

1.5.1 PC Biosynthetic Pathways: There are 2 main biosynthetic pathways for PC synthesis; the Kennedy pathway as well as PE methylation. In addition, PC can be synthesized by reacylation of LPC, or a base-exchange reaction where the serine headgroup of phosphatidylserine is exchanged for choline (279). However, the next section will concentrate only on the 2 main pathways.

A) The Kennedy Pathway: The Kennedy pathway, also known as the CDP-choline pathway, relies on the dietary intake of choline. As described in section 1.4.3, in mammals, the Kennedy pathway is responsible for PC synthesis in all nucleated cells (237). In the cell, choline kinase (CK) phosphorylates choline and commits it to PC

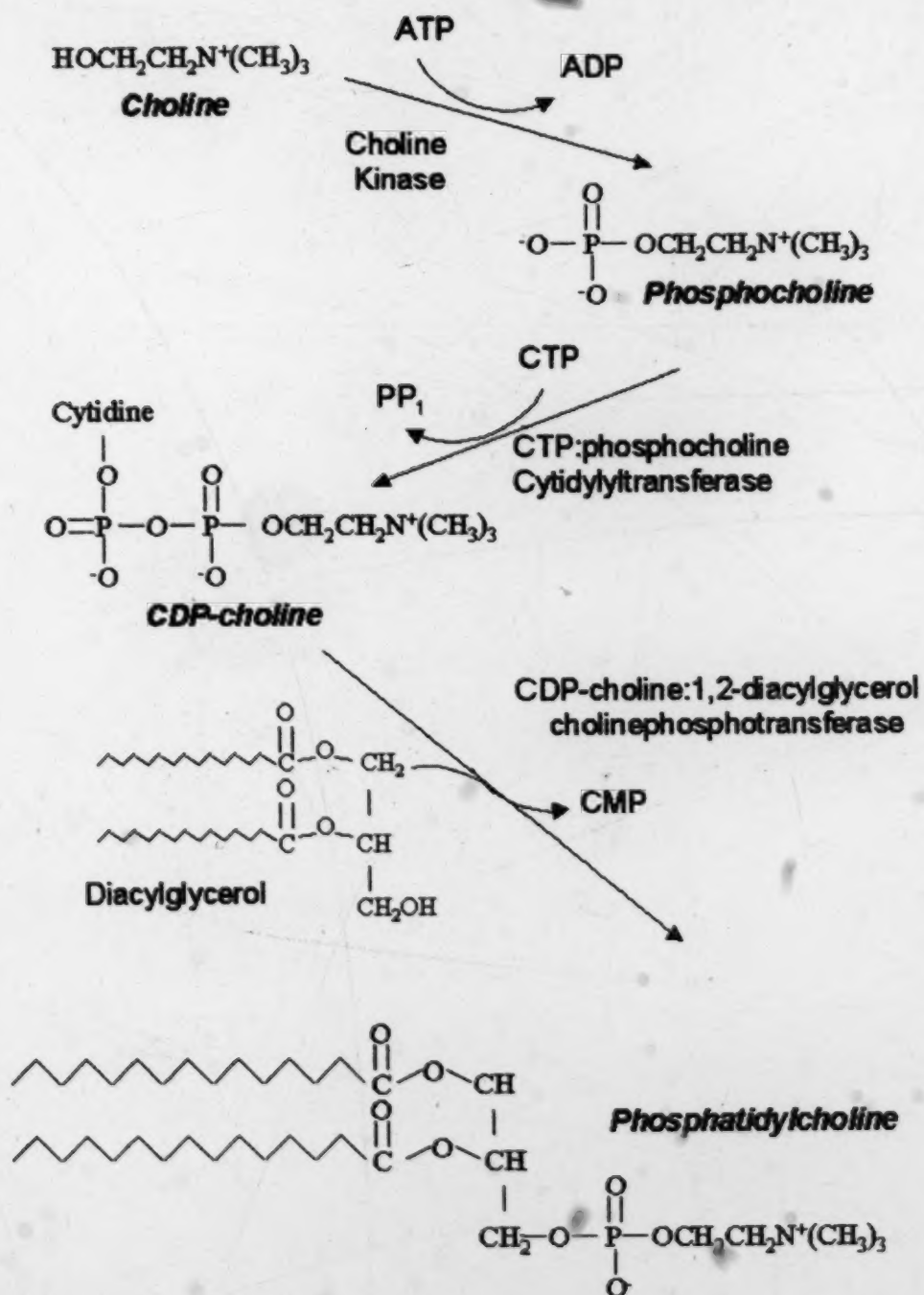
synthesis (Fig. 1.10) (280). CK is a cytosolic protein whose activity requires Mg^{2+} , and it uses ATP as the phosphate donor (280). Enzymatic activity has been purified to apparent homogeneity from rat kidney, liver and brain (280). All evidence points to there being several isoforms of this enzymatic activity (280). Recently, the genes for the murine CK isoforms α and β were characterized and it appears that $CK\alpha$ also undergoes alternative splicing (281). It is hypothesized though that there are even more isoforms of this enzyme in the mouse (281). In addition, the substrate specificity of CK is not limited to choline. This same enzyme phosphorylates ethanolamine in the CDP-ethanolamine pathway where the final product is PE (280, 281). Although CK is the first step of the Kennedy pathway, it is not rate limiting (280, 281). However, recent data suggests that it is potentially a slow step, and it is regulated (280). As well, CK has been shown by several studies to be induced by a number of mitogenic stimuli (282, 283).

The second, rate limiting step of PC synthesis is catalyzed by CTP: phosphocholine cytidylyltransferase (CT) (279) and there are three known isoforms; α , β_1 and β_2 (284). The two β isoforms are splice variants of each other where β_1 is missing the last 49 amino acids of the C-terminus of β_2 (284). The β isoforms were discovered only recently and so little is known about these two isoforms. Still, the sequence identity of the membrane binding site, as well as the catalytic domain of these two isoforms are nearly identical with $CT\alpha$ and so it is hypothesized that many of the characteristics of $CT\alpha$ run true for the β isoforms (284, 285).

$CT\alpha$ in its inactive form exists as a soluble homodimer in the cytosol or within the nucleus (279). Upon activation it translocates to a membrane where it acts (279). The site of activated $CT\alpha$ is a controversial topic. The N-terminus of $CT\alpha$ contains a nuclear targeting sequence (286), and subcellular fractionation as well as immunolocalization studies have shown it to be found in the nucleus and on the nuclear membrane upon activation (287-289). However, another study has shown that $CT\alpha$ is also shuttled from the nucleus to the cytosol and the ER membrane (290). On the other hand, the two β isoforms lack the nuclear targeting sequence and have been located in the

Figure 1.10: PC Biosynthesis by the Kennedy Pathway

PC synthesis via the Kennedy pathway relies on the intake of exogenous choline into the cell. The choline is then phosphorylated by the actions of choline kinase. The rate-limiting step is catalyzed by CTP: phosphocholine cytidyltransferase where the phosphocholine is activated to CDP-choline. Lastly, a DAG moiety is added in exchange for CMP to produce PC. This last step is catalyzed by CDP-choline: 1, 2-diacylglycerol cholinephosphotransferase (figure adapted from reference 279).



cytosol (inactive form) and on the ER membrane (activated) (284, 285). There are also a few studies where some CT activity was localized at the Golgi complex (291, 292).

Because CT is the rate-limiting step of this pathway, it is very sensitive to regulatory mechanisms. CT α is regulated transcriptionally by Sp1, Sp2, Sp3 (293, 294), Ap1 (293), SREBP (295), tumor necrosis factor- α (296) and transcriptional enhancer factor-4 (297). Also, stimulation of quiescent cells to grow results in a 4-fold increase of CT α mRNA levels (298). Finally, mRNA stability is also a known method of CT α regulation in the lung (299). The majority of research has however concentrated on the mechanisms that regulate the translocation of CT α from its inactive soluble form to the membrane bound form.

Firstly, CT activation is strongly regulated by lipids (279). Treatment of cultured cells with phospholipase C stimulates translocation of CT activity to the membrane fraction (300). DAG and free fatty acids are particularly potent activators of CT (279). Additionally, exposing cells to CD conditions where PC levels are constrained also stimulates CT activation (301). In contrast, elevating PC concentrations by either treatment with LPC (302) or expression of PEMT (303) generally inhibits CT activation. Secondly, the extreme C-termini of both CT α and β_2 both contain many different phosphorylation sites (304, 284). In CT α studies it has been shown that highly phosphorylated forms of this isoform tend to be in the inactive state, while unphosphorylated forms were membrane bound (279). These trends have not yet been shown for CT β_2 but are hypothesized to be similar (284). Therefore, it is strongly believed that CT activation is regulated by phosphorylation events. Still, it has been difficult to show that these changes in CT α phosphorylation are a direct prerequisite for translocation and therefore, the exact regulatory role phosphorylation plays on CT activity is not yet fully elucidated (305).

The final step of the Kennedy pathway is the addition of DAG to the CDP-choline moiety to form the final product, PC (279). This step is catalyzed by the membrane bound CDP-choline: 1,2-diacylglycerol cholinephosphotransferase (CPT) of the Kennedy pathway (278, 279). Due to the highly hydrophobic nature of this enzyme, purification attempts have not yet been successful and consequently little is known about this enzyme

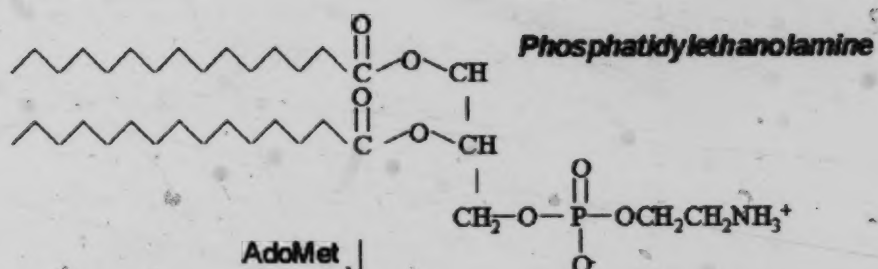
other than the phospholipid environment greatly affects CPT activity (278). Subcellular fractionation studies localize CPT to the ER. However, some activity has also been found at the Golgi, nucleus and mitochondrial fractions depending on the methodology used (278, 291). Very recently two forms of human CPT were cloned, one which has both choline and ethanolaminephosphotransferase activities (hCEPT) (306) and one which only recognizes CDP-choline as a substrate (CPT1) (307). CEPT is expressed ubiquitously in all tissues, and it has a predicted mass of 46.6 kDa (306). CPT1 expression on the other hand is most predominant in testis, colon, heart, prostate and spleen and has 60% identity with CEPT (307). Both enzymes are able to reconstitute PC synthesis in yeast (306, 307). With the recent cloning of these two enzymes, perhaps some progress can now be made with regards to understanding this final step of the Kennedy pathway.

B) PE Methylation: Of the two PC biosynthetic pathways, PE methylation is the more ancient. Generally, prokaryotes do not contain PC in their cellular membranes however in the few species that do, it is synthesized mainly by PE methylation (308). Yeast is capable of synthesizing PC via both PE methylation and the CDP-choline pathway (309). Still PE methylation is believed to be the predominant pathway utilized (309). For yeast grown in the absence of choline, the CDP-choline pathway functions mainly to recycle choline catabolized from the PC synthesized via PE methylation (278). Unlike the mammalian system, PE methylation to PC is catalyzed by two enzymes in yeast (309). The *PEM1/CHO2* gene product catalyzes the first methylation reaction converting PE to PMME, while *PEM2/OPI3* encodes the enzyme that catalyzes the production of phosphatidyl dimethylethanolamine (PDME) and PC (309).

In rodents it is firmly established that PEMT alone catalyzes all 3 methylation reactions (Fig. 1.11) (310). Rat PEMT is a 22.3 kDa protein with 4 predicted transmembrane regions and activity has been detected primarily in the ER, and the mitochondrial associated membrane (MAM), while a small amount of activity is also found at the Golgi (311, 291). In rodents, significant PEMT activity has only been detected in hepatic tissues where it is hypothesized to contribute 20-40% of total PC produced (308, 238). In other tissues, PEMT activity is only 2-6% of that found in liver

Figure 1.11: PC Biosynthesis Via PE Methylation

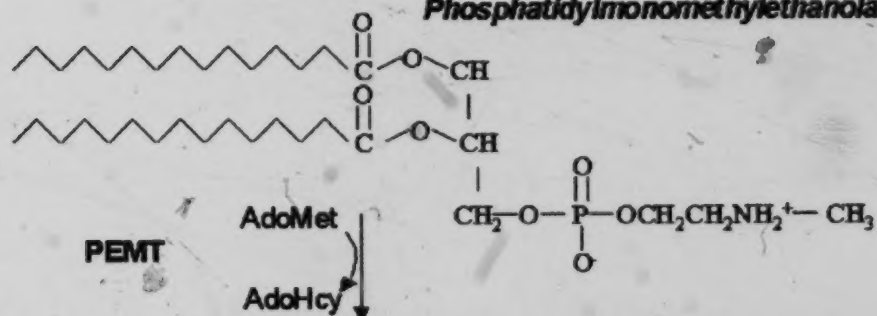
PEMT methylates phosphatidylethanolamine 3 times consecutively to produce phosphatidylcholine. AdoMet serves as the methyl donor, and 3 AdoHcy molecules are produced with each PC molecule made. In mammals studied so far, PEMT activity is significant only in the liver (figure adapted from reference 237)



PEMT

AdoMet

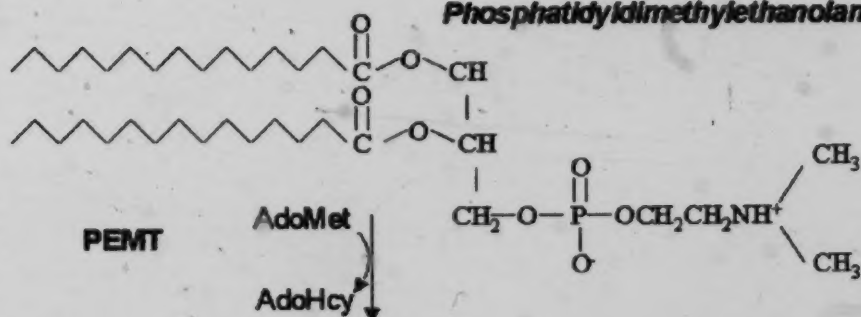
AdoHcy

Phosphatidylmonomethylethanolamine

PEMT

AdoMet

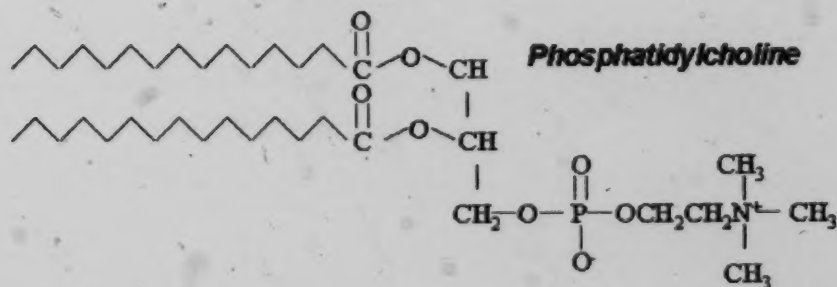
AdoHcy

Phosphatidylidimethylethanolamine

PEMT

AdoMet

AdoHcy

Phosphatidylcholine

and therefore is not believed to contribute significantly to total PC synthesis (308). Recently, 3 novel cDNAs were cloned for human PEMT (312). These cDNAs differed in the 5' untranslated region, and it was later shown that these 3 different regions are a result of alternative splicing (313). Expression profile analysis of PEMT transcripts of human tissues, indicated that PEMT expression occurs in fetal and adult liver, and to a much smaller degree, heart and testis (313). Profiles of PEMT transcripts in the liver, heart and testis were different as a result of alternative usage of these three 5' untranslated exons (313). These results suggest that the expression profile of PEMT in humans is regulated by the alternative splicing of the PEMT transcript (313).

With regards to regulation of PEMT activity very little is known. Research on PEMT is often difficult for a number of reasons. Firstly, there are no known immortalized cell lines that naturally express PEMT (D.E. Vance, unpublished results). This limits work to the whole animal, primary hepatocytes or stable expression in hepatoma cell lines. Additionally, PEMT activity in primary hepatocytes is not stable but lost over time, which could potentially interfere with any regulation studies (Chapter 4). Secondly, although PEMT was purified in 1987 by Neale Ridgway (314), there has been little success in repeating his purification scheme (D.E. Vance, unpublished). PEMT is so hydrophobic that proper solubilization is critical for maintenance of activity during the purification process (311, 314).

Little work has been done on the transcriptional regulation of PEMT however, there are two known stimuli that result in changes in PEMT mRNA levels. PEMT expression does not begin in rodents until after birth, in correlation to a decrease in the liver growth rate (315). Furthermore, after a partial hepatectomy PEMT expression is temporarily lost, while the liver regenerates, and CT expression is upregulated (316). Finally, a number of other studies have shown an inverse correlation between PEMT expression and the growth rate of rat liver or cells (317, 318). The mechanism, as well as the reasons for the loss of PEMT expression in response to hepatic growth rate is unknown. One possibility is that the tissue recognizes that PEMT is a less efficient source of PC than the Kennedy pathway. Stable expression of PEMT in temperature sensitive CT mutant CHO cells did not rescue them from apoptosis at the restrictive temperature (319). Recent work by Waite and Vance showed that the PEMT expressors

did not produce sufficient PC to maintain the growth of these cells since PEMT cannot use recycled choline and relies completely on the rate of PE production (210).

The second stimulus to induce changes in PEMT expression is long term choline deficiency (320). In a CD state such as starvation, PE methylation is the only source of choline and PC for the body. Studies in rats have shown that although choline deficiency induces a fatty liver and decreased lipoprotein secretion, the animal survives, probably because hepatic PEMT provides enough PC to maintain life (321). One study demonstrated that after a 2 day CD diet, PEMT activity (utilizing endogenous lipid substrate) doubled. Immunoblots using an antibody raised against the purified PEMT protein indicated no change in PEMT levels, suggesting that the changes were not due to a change in protein expression (322). Lipid analysis suggested that the increase in PEMT activity was actually due to an increase in the concentration of PE (322). Increases in PEMT mRNA and protein levels are only observed after a 3 week CD diet (320). The reasons for this slow response have not yet been fully examined.

Most studies examining the hormonal regulation of PEMT activity have in many cases proven inconclusive (308). Most studies investigating the phosphorylation of PEMT were performed on what was believed to be the purified PEMT, but was later shown to be a contaminant (323-326). Ridgway and Vance showed that although PEMT was phosphorylated *in vitro* by a cAMP dependent kinase, they were not able to repeat this result in intact hepatocytes (327).

The option that some type of post-translational modification regulates PEMT activity, is still a possibility. The majority of PEMT activity is localized at the ER (311). When a polyclonal antibody was raised against the predicted C-terminal 12 residues of PEMT, this antibody was able to only cross-react with activity localized at the MAM fraction (311). The MAM is a specialized extension of the ER that is in close proximity with the mitochondria (328). Although the function of this fraction is not known, it is enriched with lipid synthesizing enzymes (328). The mechanism causing this change at the C-terminus of PEMT resulting in the distribution of PEMT activity between the ER and the MAM has not been determined. Also, the purpose of having 2 separate pools of PEMT activity is also not understood. Originally, it was hypothesized that these two PEMT activities were separate isoforms expressed by two genes. The development of the

Pemt^{-/-} mouse however resulted in complete loss of all PEMT activity, quelling that theory (310).

The only well established regulatory mechanism for PEMT activity is substrate availability. Incubating primary rat hepatocytes with either excess ethanolamine, or Met results in a doubling in the rate of PE methylation to PC (329, 238). As well, the ratio of AdoMet/AdoHcy is an extremely potent regulator of PEMT activity (308). Perfusion studies on rat livers where the AdoMet/AdoHcy ratios were changed from 5.6 to 0.3 resulted in a >99% loss of incorporation of radiolabeled Met into PC (330). Also, 3-deazaadenosine (DZA) is a potent inhibitor of AdoHcy hydrolase (Fig. 1.5) and treatment of cells with DZA results in an accumulation of both AdoHcy as well as deaza-AdoHcy (331, 332). Experiments where 20 mg of DZA were injected into rats resulted in a 90% decrease in incorporation of Met radiolabeled PC (332). Treatment of primary rat hepatocytes with DZA has also been shown to reduce PEMT activity greater than 95% (333).

1.5.2. The Function of PEMT: Choline is readily accessible in all diets and so it is not clear why PEMT activity has survived evolutionary selection. Furthermore, it is odd that significant PEMT activity is detected only in the liver. Trying to elucidate the role of PEMT derived PC in mammalian systems has been a challenging task. The only proven role of PEMT is that it serves as a back up source of PC during times of choline deficiency (239). This role was firmly established with the development of the *Pemt*^{-/-} mouse. When these mice were put on a CD diet for 3 days they died (239). Tissue analysis demonstrated that the liver had undergone extensive damage, was enlarged and lipid laden (239). Phospholipid analysis of the tissues revealed that although most tissue PC levels were normal, the liver had undergone a 50% loss of this essential phospholipid. Plasma lipoprotein levels were essentially non-existent, and the gall bladder was engorged with bile (239). The *Pemt*^{+/+} animals put on this same diet suffered none of these ill effects proving PEMT activity is required for survival during choline deficiency (239).

The liver specific location of PEMT suggests it potentially contributes PC to bile or lipoprotein secretion. Experiments have been performed to verify whether or not

either of the 2 PC biosynthetic pathways is sufficient for bile secretion, and which is responsible for synthesizing the unique acyl chain composition of biliary PC (palmitate, 16:0, at the *sn-1* position and linoleate, 18:2, at the *sn-2* position) (334). These studies were performed investigating only one pathway at a time. This was achieved by feeding normal mice a CD diet, or using the *Pemt*^{-/-} mouse on a control diet. These studies showed that either PE methylation or the Kennedy pathway could provide PC for bile secretion, however biliary PC was decreased in the case of the animals relying on PEMT activity alone (334). Nevertheless, these studies did not address whether or not PEMT derived PC is specifically targeted for bile secretion under normal dietary circumstances and this question remains to be answered.

Studies exploring the targeting of PEMT-derived PC for VLDL secretion have so far been inconclusive. The studies by Yao described in Section 1.4.3D demonstrated that in the rat, PEMT derived PC on its own was insufficient to maintain normal VLDL secretion (240, 241). However, under normal dietary conditions, the Kennedy pathway is the predominant source of PC in the liver (237, 279). With choline deficiency, PEMT derived PC needs to be directed to where it is needed the most. Since this is the secondary pathway it comes as no surprise that VLDL secretion was reduced as a result of the diet. Regardless, the clear conclusion from these studies was that in the absence of 1 of the PC biosynthetic pathways, the other was required for continued secretion of VLDL (240). Still, none of these studies addressed whether or not PEMT derived PC was specifically targeted for VLDL secretion.

Inhibition of PEMT activity by treating primary rat hepatocytes with DZA did not diminish VLDL secretion from these cells (333). Later, it was shown that although PEMT activity was inhibited >95%, the small amount of PEMT derived PC that was produced, was targeted specifically for VLDL secretion (335). Treatment of rat primary hepatocytes with bezafibrate, a known inhibitor of PEMT activity was shown to decrease the secretion of apoB48 containing VLDL. Instead, more apoB48 was secreted on HDL sized particles (336). Finally, the lipoprotein profiles of *Pemt*^{-/-} mice are normal as long as their diet contains choline, which suggests that PEMT derived PC is not required for normal VLDL secretion (310). However, it is still not well established whether or not

PEMT derived PC is targeted for VLDL secretion under choline supplemented, normal genotypic conditions.

Another potential role for PEMT is the synthesis of distinct species of PC. A number of studies in whole animals, primary hepatocytes, as well as McArdle cells demonstrate that PEMT tends to produce PC containing more long chain, polyunsaturated fatty acids (337-340). This is because PE is usually composed of long chain fatty acids, and the PEMT derived PC simply mimics its precursor (338). This specificity could play an important role with regards to signal transduction, as well as membrane structure and fluidity (13, 11). However, in one of the studies it was demonstrated that PEMT derived PC was rapidly remodeled such that it resembled the majority of the other PC species (339). Therefore, it is still unknown whether this is a specific function of PEMT. The *Pemt*^{-/-} mice are physiologically normal if fed a chow diet, suggesting that remodeling of PC from the Kennedy pathway is sufficient to fulfill this potential role.

Finally, some studies have been performed in rats, investigating the regulation of PC biosynthesis by the 2 pathways as a function of gender. Interestingly, the conclusions of all of these studies suggest that females rely on PEMT derived PC more so than males. This was demonstrated by showing differences in the fatty acyl composition of PC in the livers of males and females, as well as microsomal and whole animal radiolabeling studies (337, 341, 342). In addition, male rats have been shown to be far more sensitive to CD diets and showed more pronounced effects compared to females (343, 344). Interestingly, females could revert to the patterns observed in males if they were fed excessive choline for sometime prior to the CD diet (344). The reason why female rats would be more reliant on PEMT derived PC is unknown. Perhaps it has to do with their role in reproduction and lactation. During lactation and pregnancy, females need to produce excessive quantities of PC and choline (345). An ability to regulate the two PC synthetic pathways may be a requirement for this function.

With the development of the *Pemt*^{-/-} mouse it became clear that under normal dietary conditions, PEMT activity does not seem to be required for anything in particular (310). Obviously, PC is such an important molecule that the body has several systems to backup any loss of PC synthesis. In addition to the 2 biosynthetic pathways, there are, as explained earlier, 3 isoforms of the Kennedy pathway rate limiting enzyme-CT.

Therefore, what the function of PEMT derived PC is when there is sufficient choline available is still unknown. Hopefully more in-depth analysis of these animals under a number of different stress conditions will help to elude the role of this unusual enzyme.

1.6 Thesis Objectives:

PEMT activity is a secondary source of PC in the body and it appears that it has been maintained in higher eukaryotes for the specific purpose of providing PC during times of dietary stress. Nevertheless, the role of PEMT derived PC, when choline is amply provided by the diet, is not at all known. One possibility is that PEMT derived PC is simply recycled to choline, which is then channeled through the CDP-choline pathway. Perhaps, PEMT contributes PC to the general phospholipid pool, and it is targeted to where it is needed most.

This thesis attempts to answer a number of questions regarding PEMT activity. Firstly, "Does PEMT derived PC play a role in VLDL secretion?" To answer this question the *Pemt*^{-/-} mouse model is used to analyze any changes in plasma lipoprotein levels as a function of PEMT activity. Previous studies with this mouse have shown that PEMT activity is not required for VLDL secretion. However, these studies did not take into account important factors that regulate the synthesis and secretion of these particles, two of which are gender and diet. Therefore, we examine the role of PEMT derived PC in VLDL secretion with the challenge of a HF/HC diet, and in the 2 sexes. Also, hepatocytes isolated from *Pemt*^{-/-} and ^{+/+} mice are used as a model to answer this question. Primary hepatocytes are an important model for lipid and lipoprotein research since they retain many of the liver's functions, but in an isolated setting without any interfering factors from the rest of the body.

Secondly, this thesis poses the questions, "In a choline supplemented mouse, is PEMT activity regulated by factors such as diet or gender? Is the PC produced targeted for a specific pool such as bile or lipoprotein secretion?" The studies performed to answer these questions are also an attempt to understand the observations made from the first set of studies. Here, the whole animal is used with or without the challenge of a HF/HC diet. PEMT activity and intracellular distribution is examined in the livers of

these animals. Furthermore, the effects of diet and gender on the Kennedy pathway are also analyzed. Finally, the targeting of PEMT derived PC in the whole animal is studied by injecting a radiolabeled PEMT substrate into the mouse and looking at radiolabeled PC in the plasma, liver and bile. Using the information attained from this work, we hope to gain an understanding of the role of PEMT derived PC in the total scheme of PC metabolism.

Finally, as a methyltransferase, PEMT produces AdoHcy, the precursor for the cardiovascular risk factor Hcy. PEMT is a unique methyltransferase in that it performs 3 consecutive methylation reactions for each final PC molecule made. Considering that the liver synthesizes an enormous quantity of PC to fulfill the needs of membrane integrity, bile and lipoprotein secretion, and that PEMT makes up to 40% of that PC, this potentially results in a great deal of Hcy produced. Therefore, we again use the *Pemt*^{-/-} mouse and ask the question "Does PEMT activity contribute significantly to total plasma Hcy concentrations?" Little is understood with regards to the source of plasma Hcy, particularly the source of increased Hcy observed in patients at risk for cardiovascular disease. By finding a change in Hcy concentrations in *Pemt*^{-/-} mice, we come closer to understanding fully the metabolism of this deleterious molecule.

In summary, this thesis looks at the contribution of PEMT activity towards the development and the advancement of the atherosclerotic plaque. In trying to understand the effect of PEMT activity on lipoprotein metabolism we take into account other cardiovascular disease risk factors. Although there is some knowledge with regards to the synthesis and secretion of a VLDL particle, how factors such as gender and diet specifically affect this process is not well understood. The potential function of PEMT as a regulator of plasma Hcy levels also poses a possible role for PEMT to the development of cardiovascular disease. Understanding the regulation of PC metabolism as a function of diet and gender may also lend information as to how these factors regulate plasma Hcy levels.

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Chapter 2

**A Diet and Gender Specific Role for
Phosphatidylethanolamine *N*-methyltransferase
Derived Phosphatidylcholine in the Secretion of
Very Low Density Lipoproteins in Mice**

2.1 Introduction

PC is synthesized by a number of pathways including the Kennedy pathway, phospholipid methylation, and acylation of LPC. The Kennedy pathway is the major pathway for PC synthesis in all mammalian tissues, and is dependent on the intake of dietary choline (1). In the liver an additional pathway for PC production is catalyzed by PEMT which transfers 3 consecutive methyl groups from AdoMet to PE (2).

The liver specific location of PEMT suggests it may have a role in either bile secretion, or VLDL secretion. All lipoproteins consist of an inner core of insoluble neutral lipid surrounded by a soluble outer monolayer consisting of phospholipid and protein. PC is the primary phospholipid of all classes of lipoproteins (3) and is required for the secretion of VLDL; other phospholipid species cannot substitute (4-7). The Kennedy pathway is required for VLDL secretion from the liver since rats put on a CD diet for 3 days were found to have a 6-fold increase in liver TG, as well as decreased plasma TG levels (4, 7). The role of PEMT derived PC however is not as well established.

Treatment of rat hepatocytes with DZA, an inhibitor of all methylation pathways that utilize AdoMet, inhibited PEMT activity >95% however did not decrease VLDL secretion (8). Later a conflicting observation showed that phosphatidylserine (PS) derived PC is targeted for VLDL secretion, and secretion of this PC pool was not inhibited at all by DZA treatment (9, 10). In these experiments, the radiolabeled PS derived PC was produced via a decarboxylation step of PS to PE followed subsequently by PEMT methylation to PC, suggesting that inhibition of PEMT activity was not complete. More recent studies using bezafibrate to inhibit PEMT activity in rat hepatocytes showed a shift of apoB48-containing lipoproteins to higher density (11). Unfortunately, the inhibitors used in all these studies are non-specific and are known to inhibit many other enzymatic activities (8, 11).

With the production of the *Pemt*^{-/-} mouse, a model now exists to assess the role of PEMT in VLDL secretion more directly (12). *Pemt*^{-/-} mice appear normal except when fed a CD diet where PC biosynthesis is compromised and the mice develop severe liver failure (13). We now report studies on lipoprotein secretion from mice fed either a chow or HF/HC diet. Male, but not female, *Pemt*^{-/-} mice exhibit a defect in VLDL and

apoB100 secretion. In contrast, the female *Pemt*^{-/-} mice demonstrated a 40% decrease in plasma cholesterol and PC. Lipid analysis of the plasma lipoproteins indicated these decreases observed in the females to be changes in HDL. Furthermore, normal hepatic and biliary PC in the *Pemt*^{-/-} mice suggests that lipoproteins have lower priority with regards to PC distribution.

Previous choline deficiency and radiolabeling studies with rats have shown that females produce, and rely on PEMT derived PC more than males (14, 15). This dependence on PEMT can be reverted to a pattern similar to males if they are given excess choline prior to a CD diet (16). Nevertheless, it is surprising that the Kennedy pathway is unable to compensate for the PEMT deficiency with the dietary conditions utilized in this study.

2.2 Materials and Methods

2.2.1 Materials: The semi-purified diet lacking a fat source was attained from ICN. The regular rodent chow was obtained from LabDiet (PICO Lab Rodent Diet 20). Diagnostic kits for total cholesterol, total triglycerides, total glycerol and aspartate and alanine aminotransferases were purchased from Sigma. Plasma phospholipids and the free cholesterol diagnostic kits were purchased from Wako Chemicals. Silica gel G60 plates for thin-layer chromatography (TLC) were from Merck, while the PC, PE, PS and TG (triolein) standards were from Avanti Polar Lipids. The SM and LPC TLC standards were purchased from Sigma. The sheep anti-human apoB antibody was purchased from Boehringer Mannheim and the rabbit anti-human apoA1 antibody was from Biodesigns. Both the donkey anti-sheep and the goat anti-rabbit conjugated horseradish peroxidase secondary antibodies were purchased from Pierce as well as the enhanced chemiluminescence detection system. Triton WR 1339, cholesterol and Protein A Sepharose CL-4B were purchased from Sigma. Finally, all other chemicals and reagents were purchased from standard commercial sources.

2.2.2 Care and Feeding of Mice: The *Pemt*^{-/-} mouse colony had a mixed genetic background of 129/J and C57BL/6 (12) and was maintained via homozygous breeding in

a reversed 12-hr light/dark cycle. At the age of 12-14 weeks the animals were fed *ad libitum* either a control diet of regular rodent chow, or a HF/HC diet. The HF/HC diet was made as previously described (17). Briefly, it consisted of 80% (w/w) semi-purified diet lacking a fat source, 19% (w/w) olive oil, 1% (w/w) linseed oil (a source of essential fatty acids) and 1% (w/w) cholesterol. In contrast the known composition of the rodent chow was 4% fat and 0.1% cholesterol. During the diet the animals were held in regular cages with bedding, in a non-reversed 12-hr light/dark cycle. Following the 3 weeks, the animals were fasted overnight, weighed and sacrificed. Blood, bile and tissues were collected and processed as described below.

2.2.3 Protein, and Lipid Analysis of Livers: After extirpation, the livers were weighed, homogenized in 3 ml of homogenization buffer (50 mM Tris-HCl, pH=7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT and 0.1 mM PMSF) and sonicated for 20 seconds. Protein was determined using the Coomassie Plus protein protocol from Bio-Rad, which is based on the Bradford assay (18). Bovine serum albumin (BSA) was used as a standard for all protein assays. Lipids were extracted from the liver homogenate and separated by TLC (19). Phospholipids were separated using a developing solvent of chloroform/methanol/acetic acid/water (25:15:4:2) until the solvent reached halfway up the plate. The plates were removed, solvent evaporated and the neutral lipids were separated using a developing solvent of heptane/diisopropyl ether/acetic acid (60:40:4) which migrated to the top of the plate. Lipid bands were visualized with iodine vapour, and the bands of interest were scraped and analyzed. Phospholipid mass was measured by analyzing phosphate content using the Malachite Green lipid phosphorus assay (20). TG mass was determined by the hydroxylamine method (21).

2.2.4 Lipid Analysis of Plasma and Bile: Blood was collected from each mouse via the lower *vena cava* in the presence of trace amounts of 250 mM EDTA, and plasma was isolated by centrifugation. Sodium azide was added to all plasma samples to a final concentration of 0.01%. Total cholesterol for both plasma and bile was measured using the Sigma Infinity Cholesterol Reagent modified to a microtiter plate format, and plasma unesterified cholesterol was analyzed by the Wako Free Cholesterol C kit also in a

microtiter plate format. Plasma CE was calculated by subtracting the unesterified cholesterol concentrations from the total cholesterol. Plasma TG was measured using the Sigma Triglyceride GPO Trinder kit (protocol 337-B).

The plasma from 3-5 animals was pooled and separated into the various lipoprotein fractions using high performance liquid chromatography (HPLC) with an Amersham Pharmacia Biotech Superose 6 column attached to a Beckman Systems Gold or Nouveau Gold apparatus. In-line assays for glycerol (Sigma Infinity Triglycerides Reagent), total cholesterol (Sigma Infinity Cholesterol Reagent), or choline-derived phospholipids (Phospholipid B, Wako) were performed during the HPLC separations as previously described (13).

Phospholipids were extracted from 100 μ l of plasma, or 4 μ l of bile from each animal using a modified Bligh-Dyer protocol (19) and separated by TLC using the phospholipid solvent system described above. The phospholipids were scraped from the plates and measured by the Malachite Green lipid phosphorus assay (20).

2.2.5 Measurement of Plasma Aminotransferases: Following isolation of plasma, aspartate aminotransferase and alanine aminotransferase activities were measured as previously described (22, 23) using the Sigma GP and GO transaminase kit.

2.2.6 Isolation and Western Blots of Plasma Apolipoproteins: The plasma from 2-3 animals was pooled to a final volume of 200 μ l, to which was added 1.3 ml of 4.151 M KBr to a final density of 1.32 g/ml. This mixture was then put at the bottom of a 5 ml Beckman Quickseal tube, and overlaid with 0.9% NaCl. The samples then were centrifuged in a VTi 65.2 rotor at 416 000 g for 1 hr as previously described (24, 25). Ten, 0.5 ml fractions from each sample were isolated, with densities ranging from 1.200-1.010 g/ml. To each fraction, 200 μ l Cab-O-Sil slurry was added (0.5 g/10 ml PBS) and mixed for 15 min at 4°C (26). After, the Cab-O-Sil was pelleted by centrifugation at maximum speed for 5 min in a microfuge, and rinsed 2 times with PBS. The pellets were boiled in 1x Laemmli sample buffer (27) and proteins were separated on a 3-15% continuous gradient SDS-PAGE gel and transferred onto nitrocellulose membranes.

Western blots were performed against apoB; the membranes were stripped and blotted for apoA1. Band quantitation was performed using Image Gauge v3.0 software by Fuji.

2.2.7 Analysis of ApoB Secretion *In vivo*: *Pemt*^{-/-} and ^{+/+} males were put on HF/HC or chow diets for 3 weeks. Subsequently, the animals were fasted overnight and in the morning each was injected with a total of 200 μ l of PBS containing 10% Triton WR 1339 (v/v) and 500 μ Ci [³⁵S]-Promix (Amersham-Pharmacia) as previously described (28). After 1 hr, the animals were sacrificed and blood was collected with EDTA by cardiac puncture. To 100 μ l of total plasma, 400 μ l of PBS, 55 μ l of 10x immunoprecipitation buffer [1.5 M NaCl, 0.5 M Tris pH 7.4, 50 mM EDTA, 5% Triton X-100 (v/v) and 1% SDS (w/v)] and 10 μ l of anti-human apoB antibody as well as benzamidine (total concentration 1 μ M) were added and mixed overnight at 4°C (25). Protein A Sepharose CL-4B was added (100 μ l of 1:1 mixture with PBS) to each sample and mixed for 1 hr at 4°C. Immunoprecipitates were pelleted by centrifugation, rinsed 2 times with 1x immunoprecipitation buffer and boiled in 1x sample buffer [125 mM Tris/HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), 10% β -mercaptoethanol (v/v), and 0.02% Bromophenol Blue] (25, 29). Samples were run on a 5% SDS-PAGE gel, and proteins were stained with Coomassie stain. The gels were soaked in Amplify, dried and exposed to film.

2.2.8 Histological Analysis: Livers were fixed in 10% formalin and sections were stained by standard protocol with hemotoxylin and eosin. In addition, livers fixed in 10% formalin were quick-frozen, sectioned and stained with Oil Red O to examine neutral lipid distribution.

2.3 Results

2.3.1 Histological Characterization of Mice as a Function of Diet: At the end of the 3-week diets, all animals were fasted overnight and the next morning were weighed and sacrificed. Both male and female *Pemt*^{-/-} animals were comparable in weight to the ^{+/+} mice (Table 2.1) and they appeared normal and healthy. However, 80%

		Body Weight (g)	Liver Weight (g)	Liver/Body (%)
Female				
Chow	+/+	22.29 $\pm$ 0.87	0.82 $\pm$ 0.023	3.70 $\pm$ 0.15
	-/-	22.64 $\pm$ 0.45	0.82 $\pm$ 0.038	3.60 $\pm$ 0.15
HF/HC	+/+	24.61 $\pm$ 2.21	0.90 $\pm$ 0.036	3.98 $\pm$ 0.15
	-/-	23.70 $\pm$ 0.47	0.96 $\pm$ 0.034	4.05 $\pm$ 0.11
Chow	+/+	24.79 $\pm$ 1.15	0.97 $\pm$ 0.046	4.01 $\pm$ 0.10
	-/-	27.04 $\pm$ 1.11	1.08 $\pm$ 0.044	3.92 $\pm$ 0.10
HF/HC	+/+	26.23 $\pm$ 1.39	1.01 $\pm$ 0.064	3.85 $\pm$ 0.08
	-/-	29.21 $\pm$ 1.74	1.27 $\pm$ 0.123	4.27 $\pm$ 0.18

Table 2.1: Mice have Normal Body and Liver Weights.

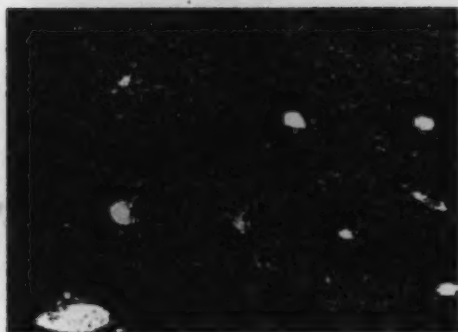
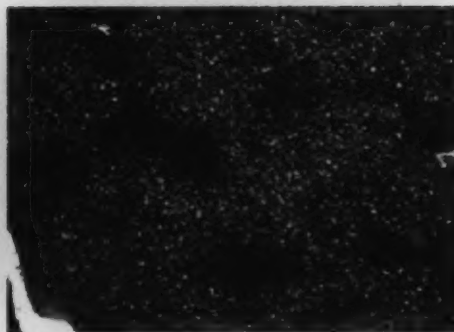
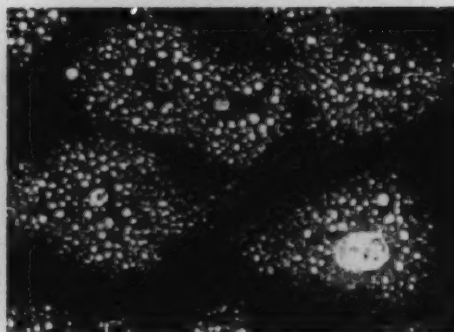
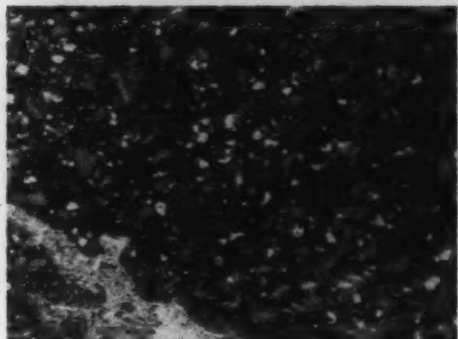
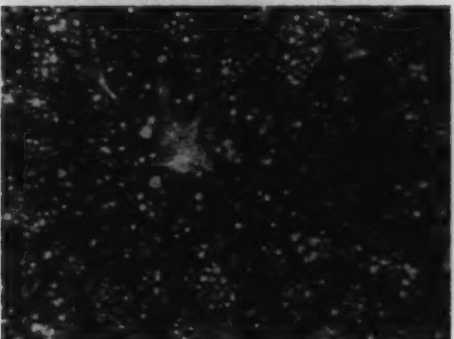
Prior to sacrifice all mice were weighed. Following sacrifice, the wet liver weight of each animal was measured as well. Shown are the mean, $\pm$ S.E.M. for 9-10 mice for each of the above conditions. The difference in liver/body % between *Pemt*^{-/-} and *+/+* males fed a HF/HC diet approached significance at a value of $P=0.059$ based on a paired Student's t-test.

of the livers from the male *Pemt*^{-/-} mice fed the HF/HC diet were granular in appearance and some were also lighter in color, suggesting an accumulation of lipid. Histological examination suggested gross vacuolarization occurring in a centrilobular pattern in approximately 50% of hepatocyte lobules in these male *Pemt*^{-/-} mice (Fig. 2.1D). Interestingly, these large vacuoles did not stain when treated with Oil Red O stain (Fig. 2.1F) suggesting they are not engorged with neutral lipid. Finally, a small, but insignificant increase in the wet liver weight of the male *Pemt*^{-/-} HF/HC fed mice, as well as a small increase in the total liver/body weight percentage was observed (Table 2.1).

Upon observation, female *Pemt*^{-/-} mice on either diet had very normal livers when compared to their *+/+* counterparts. However, histological examination suggests that the

Figure 2.1: Liver Histology of Mice Fed a HF/HC Diet.

Twelve to 16 week old animals were fed either a HF/HC diet or chow diet for 3 weeks. The animals were fasted overnight, sacrificed and livers were removed. Sections of each liver was fixed in 10% formalin and sections were sliced and stained by a standard protocol with hematoxylin-eosin (Fig. 2.1 A-D), or the sections were quick frozen and stained with lipophilic Oil Red O (Fig. 1 E, F). Shown only are sections from HF/HC fed animals. The hematoxylin-eosin stained sections are at a magnification of 10X, and the livers of 2 animals underwent histological analysis for each condition (excluding the HF/HC fed, male *Pemi*^{-/-} and ^{+/+} mice, for which 3 livers were analyzed). The liver of only 1 animal for each condition underwent the Oil Red O staining procedure and the sections are shown at a magnification of 40X. Above are representative pictures of all livers examined.

A. *Pemt*^{+/+}, Female**B. *Pemt*^{+/+}, Female****C. *Pemt*^{+/+}, Male****D. *Pemt*^{+/+}, Male****E. *Pemt*^{-/-}, Female****F. *Pemt*^{-/-}, Male**

female *Pemt*^{-/-} mice fed a HF/HC diet also have a mild form of the vacuolization observed in the males (Fig. 2.1B). The livers of the *Pemt*^{-/-} males and females fed the chow diet appeared normal as compared to *Pemt*^{+/+} mice based on histological analysis (data not shown).

Because of obvious changes in the livers from the HF/HC fed, male *Pemt*^{-/-} animals (Fig. 2.1 D, F), plasma aspartate and alanine aminotransferase activities were measured to determine if there was any liver damage in these animals. Other than 1 female (chow fed) and 1 (HF/HC) fed male, no significant increases were observed in any of the *Pemt*^{-/-} animals compared to *+/+* mice, suggesting the observed changes in plasma and hepatic lipids were likely due to the loss of PEMT activity and not due to liver damage (data not shown).

2.3.2 Hepatic TG Levels are Increased in Male *Pemt*^{-/-} Mice Fed a HF/HC

Diet: Table 2.2 outlines the lipid analysis performed on the liver tissues of all animals studied. Female *Pemt*^{-/-} and *+/+* mice both accumulated TG in their livers to the same degree compared to the chow fed females. However, the *Pemt*^{-/-} males fed the HF/HC diet had a 4-fold increase in their concentration of hepatic TG compared to their *+/+* counterparts fed the same diet. On the other hand, chow fed *Pemt*^{-/-} and *+/+* males had similar levels of TG in their livers.

Hepatic TG accumulation, liver damage and eventual death occur rapidly in the *Pemt*^{-/-} mice fed a CD diet (13). Because these animals were fasted overnight, phospholipid analysis was performed to confirm that the observations were not occurring due to a mild degree of choline deficiency. Hepatic PC levels were similar in all the *Pemt*^{-/-} animals in comparison to their *+/+* counterparts, with the exception of female HF/HC fed animals (Table 2.2). However since the PC levels in these animals were still within normal range (50-80 nmol/mg protein) this difference was not deemed to be significant. If these animals were undergoing choline deficiency, a significant decrease in hepatic PC would have been observed (13). Lipid analysis of brain, heart, kidney, and lung tissues isolated from the female animals also showed no significant changes in PC levels (data not shown).

		PC	PE	PS+PI	TG
Females					
Chow	+/+	68.08 ± 3.15	38.19 ± 1.47	11.92 ± 0.56	345.11 ± 54.75
	-/-	63.89 ± 3.53	43.08 ± 2.36	11.77 ± 0.59	324.93 ± 72.84
HF/HC	+/+	65.04 ± 2.16	31.93 ± 2.86	11.22 ± 0.79	565.86 ± 70.94
	-/-	54.82 ± 2.54	33.64 ± 2.58	10.58 ± 0.56	694.92 ± 62.77
Males					
Chow	+/+	62.33 ± 3.23	28.63 ± 3.75	17.18 ± 2.45	412.98 ± 99.99
	-/-	64.91 ± 2.63	37.49 ± 3.61	14.99 ± 1.66	519.53 ± 56.89
HF/HC	+/+	67.47 ± 2.11	34.96 ± 4.21	12.89 ± 1.71	261.05 ± 66.73
	-/-	62.06 ± 3.11	43.69 ± 4.38	11.37 ± 1.81	1124.18 ± 279.11*

Table 2.2: Hepatic Lipid Levels in *Pemt*^{+/+} and ^{-/-} Mice on Either Diet.

Lipid was extracted from liver homogenates (1 mg of protein) and separated by TLC. Phospholipid and TG mass were determined as nmol/mg of protein. Because PS and PI are not efficiently separated on the TLC plate, the results for these two phospholipids are combined. The results are the mean ± S.E.M for 8-10 mice on each diet. **P* < 0.01 based on a Student's *t*-test.

Low levels of PE accumulation occurred primarily in the male *Pemt*^{-/-} mice suggesting it would normally have been used as a substrate for PEMT activity (Table 2.2). However, these numbers were generally within normal concentrations of PE for liver (25-40 nmol/mg protein). Furthermore PS levels were also measured since it is synthesized via a base-exchange reaction with either PC or PE. Hepatic PS/phosphatidylinositol (PI) levels were also unaffected by the lack of PEMT activity

2.3.3 Biliary Lipids are Unaffected in the *Pemt*^{-/-} Mice: The quantity of bile in the gall bladder was measured using a pipetman and no differences were observed between the genotypes, even though differences were observed between the two dietary states (Table 2.3). Although decreases were observed for PC mass in the bile of *Pemt*^{-/-} mice compared to their ^{+/+} counterparts, these changes were not significant due to animal

variability. Cholesterol secretion into bile is closely associated with PC secretion (30) and trends for biliary cholesterol mimicked observations made for PC.

		Bile Quantity (μ l)	Biliary PC (nmol/ μ l)	Cholesterol (nmol/ μ l)
Females				
Chow	+/+	22.00 $\pm$ 3.84	17.42 $\pm$ 1.06	3.67 $\pm$ 0.47
	-/-	21.89 $\pm$ 2.30	17.06 $\pm$ 1.32	2.90 $\pm$ 0.23
HF/HC	+/+	15.70 $\pm$ 2.63	17.93 $\pm$ 1.44	3.67 $\pm$ 0.21
	-/-	17.70 $\pm$ 2.49	12.94 $\pm$ 1.68	2.92 $\pm$ 0.31
Males				
Chow	+/+	20.89 $\pm$ 1.89	15.96 $\pm$ 3.20	3.41 $\pm$ 0.36
	-/-	21.60 $\pm$ 0.52	14.82 $\pm$ 0.89	2.61 $\pm$ 0.18
HF/HC	+/+	13.30 $\pm$ 1.58	14.46 $\pm$ 1.55	3.05 $\pm$ 0.23
	-/-	15.10 $\pm$ 1.46	11.14 $\pm$ 1.08	2.64 $\pm$ 0.21

Table 2.3: Bile Quantity and Composition is Unchanged in *Pemt*^{-/-} Animals

Bile was collected from all animals directly following sacrifice and the volume of liquid was measured using a pipetman. Then 4 μ l of bile was extracted for lipid and separated on TLC. PC mass was assayed by the Malachite Green method. Total cholesterol was measured directly from the bile using the Infinity Cholesterol Reagent supplied by Sigma.

2.3.4 Plasma Lipids are Altered in *Pemt*^{-/-} Animals in a Gender Specific

Manner: Choline derived phospholipids (PC, SM and LPC) make up the majority of phospholipids in the plasma (31). Analysis of plasma PC in the *Pemt*^{-/-} females showed a 30% decrease under chow conditions and a 40% decrease under HF/HC dietary conditions compared to their ^{+/+} counterparts (Table 2.4). Furthermore, PC levels increased in the *Pemt*^{+/+} female mice in response to the HF/HC diet, while it stayed the same in *Pemt*^{-/-} mice compared to the chow levels. Interestingly, the chow fed *Pemt*^{-/-} male mice had normal plasma PC levels as compared to the *Pemt*^{+/+} males. In response

		PC	LPC	SM	PS+PI	PE
Females						
Chow	+/+	49.94 ± 3.34**	19.62 ± 1.70	7.44 ± 1.02	7.41 ± 0.76	2.96 ± 0.28
	-/-	33.93 ± 4.96	16.28 ± 2.03	7.97 ± 1.80	6.72 ± 0.76	3.23 ± 0.37
HF/HC	+/+	55.63 ± 3.15*	19.05 ± 1.87**	9.39 ± 1.61	8.61 ± 1.14	2.50 ± 0.11
	-/-	34.39 ± 3.71	11.33 ± 1.68	5.71 ± 1.36	5.64 ± 0.76	2.49 ± 0.23
Males						
Chow	+/+	63.89 ± 3.68	12.76 ± 0.47	4.54 ± 0.40	7.95 ± 0.82	2.96 ± 0.28
	-/-	61.67 ± 2.98	11.75 ± 0.74	5.85 ± 0.91	7.63 ± 1.19	3.23 ± 0.37
HF/HC	+/+	82.56 ± 5.28***	18.97 ± 0.74**	7.27 ± 0.41	10.77 ± 0.73	7.81 ± 0.40
	-/-	66.85 ± 3.28	11.85 ± 0.70	6.49 ± 0.75	9.42 ± 0.06	6.82 ± 0.42

Table 2.4: Plasma Phospholipids are Altered in *Pemt*^{-/-} Mice in a Gender and Diet Specific Manner.

Lipids were extracted from 100 µl of plasma isolated from the sacrificed animals and separated by TLC. Phospholipid mass (µmol/dl) was determined via a Malachite Green lipid phosphorus assay. Shown are the mean values, ± S.E.M. from 8-10 mice on each diet. * $P < 0.001$, ** $P < 0.01$, and *** $P < 0.05$ based on a Student's t-test.

to the HF/HC diet, as in the females, an increase was seen in the ^{+/+} mice but not the *Pemt*^{-/-} males (19% lower plasma PC in the *Pemt*^{-/-} versus the ^{+/+} males).

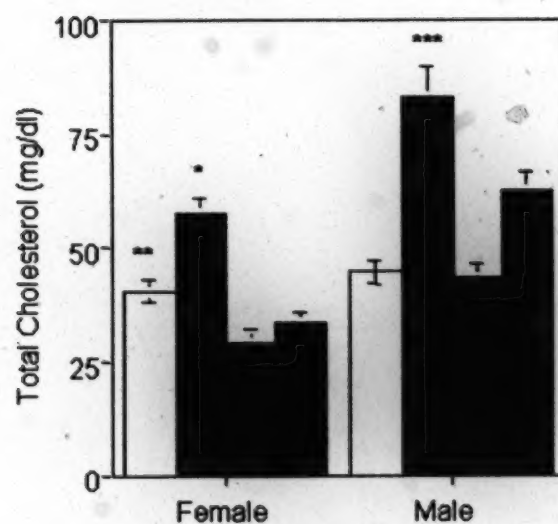
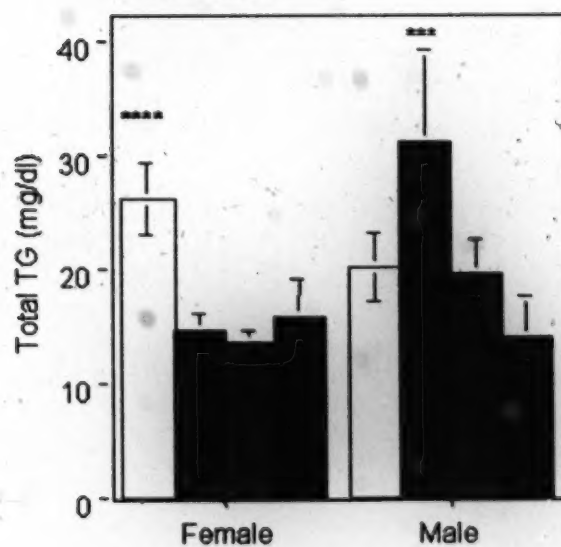
LPC levels were significantly decreased in both male and female *Pemt*^{-/-} mice under the HF/HC dietary condition only, while SM levels were not significantly changed between the genotypes for either gender (Table 2.4). Analysis of plasma PS+PI and PE also showed that these phospholipids do not compensate for the lack of choline derived phospholipids in the *Pemt*^{-/-} mice under the HF/HC dietary conditions.

Trends in plasma cholesterol and CE levels in the mice mimicked the results observed for PC. Decreases in females occurred under both chow and HF/HC fed conditions (30-42%), while only an 18% decrease was observed in the *Pemt*^{-/-} males with the HF/HC challenge (Fig. 2.2A). In all cases, the observed decrease in total plasma cholesterol was due to decreases in both free and esterified cholesterol (data not shown).

Figure 2.2: Plasma TG and Cholesterol are Altered in the *Pemt*^{-/-} Mice in a Gender and Diet Specific Manner

A. Following the 3 week diets, the animals were fasted overnight, sacrificed and plasma was isolated. Total plasma cholesterol was measured using the Cholesterol Infinity Reagent supplied by Sigma. B. True plasma TG was measured using the TG GPO-Trinder (protocol 337-B) kit from Sigma. True plasma TG was determined by subtracting the plasma free glycerol concentration from the total plasma glycerol concentration. Shown are the means $\pm$ S.E.M for 9-11 animals for each condition. * $P < 0.0001$, ** $P < 0.01$ and *** $P < 0.05$ as determined by a Student's t-test. *Pemt*^{+/+} chow fed females had significantly higher plasma TG levels compared to all the other female mice (**** $P < 0.005$), based on an ANOVA analysis.

□ *Pemt*^{+/+}, chow ■ *Pemt*^{+/+}, HF/HC ■ *Pemt*^{-/-}, chow ■ *Pemt*^{-/-}, HF/HC

A. Cholesterol**B. Triacylglycerol**

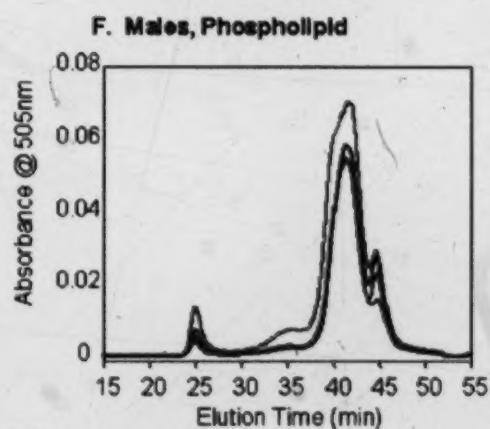
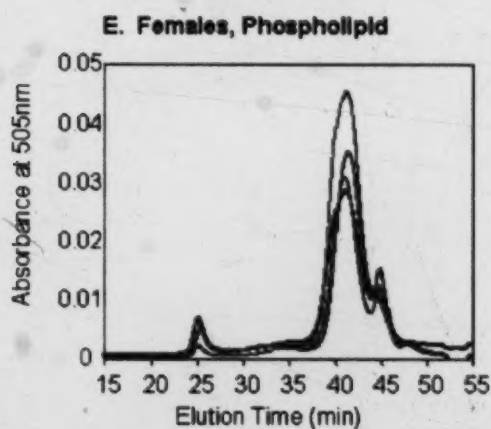
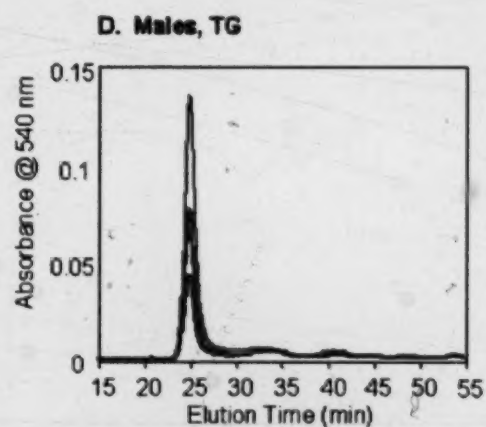
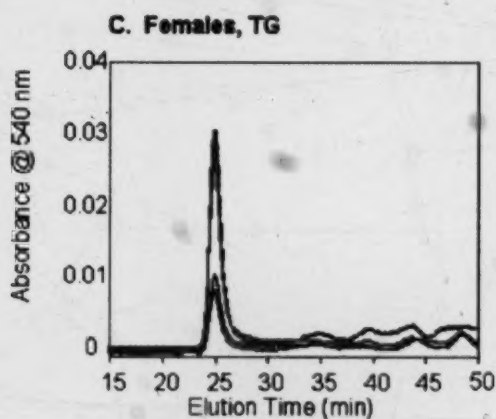
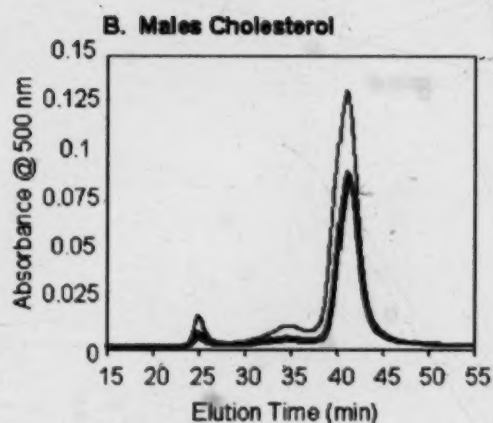
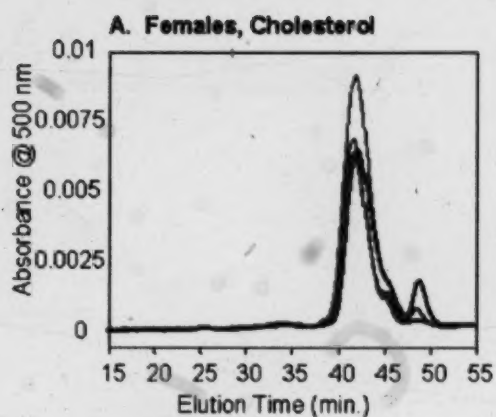
Plasma TG levels were consistently decreased in the male mice by 50% with the stress of the HF/HC diet (Fig. 2.2B). These results agree with the accumulation of TG in the hepatic tissues of these animals. Inconsistent trends in the levels of plasma TG in the females resulted in no significant difference between *Pemt*^{-/-} and ^{+/+} mice fed the HF/HC diet. The chow fed female *Pemt*^{+/+} mice had the highest level of plasma TG unexpectedly. The mechanism for this result is unknown, however, it is well established that estrogen has a role in modulating plasma VLDL, and therefore TG levels (32, 33). Differences in estrogen levels in the different female mice could explain the difficulties in getting a consistent trend in total and, as described later, VLDL TG.

The plasma from these animals was separated into its lipoprotein components by HPLC (Fig. 2.3). Elevated cholesterol and phospholipid in the HDL fractions of both male and female *Pemt*^{+/+} mice were consistently observed with the HF/HC dietary condition (Fig. 2.3 A, B, E, F). The distribution of cholesterol and phospholipids for the *Pemt*^{-/-} animals fed the HF/HC diet remained similar to the chow fed animals suggesting these mice were unable to respond to additional lipid levels in their diet to the same degree as the *Pemt*^{+/+} mice.

Consistent increases in VLDL derived TG and phospholipids was observed in the male, HF/HC fed, *Pemt*^{+/+} mice compared to the chow fed animals as well as their *Pemt*^{-/-}, HF/HC fed counterparts (Fig. 2.3 C, D, F). Again, this suggests that the male *Pemt*^{-/-} mice are unable to elevate plasma lipid levels to the same extent as the ^{+/+} animals in response to the diet. No consistent trends were observed with regards to VLDL in any of the female mice (Fig. 2.3 C, E). Nevertheless, based on the hepatic, total plasma, and lipoprotein distribution of the lipids in the *Pemt*^{-/-} males and females, it is clear that PEMT derived PC is playing a significant role in the distribution of lipids in the various lipoprotein fractions.

Figure 2.3: HPLC Separation of Plasma Lipoproteins

The plasma of 3-5 animals of each genotype, dietary and gender condition was pooled and separated into lipoprotein fractions on a Pharmacia Superose 6 column, with an in-line assay for either cholesterol, glycerol or choline derived phospholipids. Lipoproteins eluted in the following order: VLDL at 25 min., LDL/IDL at 35 min. and HDL at 40-45 min. For all of the above figures — *Pemt*^{+/+}, HF/HC; — *Pemt*^{+/+}, Chow; — *Pemt*^{-/-}, HF/HC; and — *Pemt*^{-/-}, Chow. Analysis for each condition was done twice, with the exception of cholesterol analysis of female mice, which was performed 3 times.



2.3.5 ApoB Levels are Decreased in *Pemt*^{-/-} Males Fed a HF/HC Diet: Plasma collected from 2-3 animals from each genotype, sex and diet was pooled to a total of 200 μ l, and 10 density fractions (1.200-1.010 g/ml) were isolated following ultracentrifugation. Lipid and apoprotein components in each density fraction were adsorbed by Cab-O-Sil and Western blots were performed against apoB48, apoB100, and apoA1. *Pemt*^{-/-} males fed a HF/HC diet demonstrated decreases in both apoB48 and apoB100 in the VLDL/LDL density fractions (Fig. 2.4 B, D). Total plasma apoB levels were normal for all *Pemt*^{-/-} mice fed a chow diet compared to their ^{+/+} counterparts (data not shown), and for the female ^{-/-} mice fed a HF/HC diet (Fig. 2.4 A, C). These results correlate well with what was expected based on the lipid analysis (Fig. 2.2).

Surprisingly, no significant changes in the *Pemt*^{-/-} mice were observed in the levels, or distribution of apoA1 under any conditions (Fig. 4 E, F). This contrasts with the lipid analysis where changes in HDL cholesterol and phospholipid were observed for both genders under the HF/HC condition. However, the phenomenon that HDL cholesterol is decreased with no accompanying change in total apoA1 levels has been previously observed (34).

To determine whether or not apoB secretion was decreased in the male *Pemt*^{-/-} mice fed the HF/HC diet, we performed *in vivo* labeling of the apoB proteins in the presence of Triton WR 1339 in order to inhibit the catabolism of secreted VLDL. Following the 3 week HF/HC or control chow diets, male *Pemt*^{-/-} and ^{+/+} mice were fasted overnight, and then injected with 200 μ l of 10% Triton WR 1339 (v/v) in PBS containing 500 μ Ci [³⁵S] Met/Cys. The animals were sacrificed after 1 hr and apoB100 and apoB48 were isolated from 100 μ l of plasma, via immunoprecipitation. Only apoB100 secretion was significantly decreased in the *Pemt*^{-/-} males fed the HF/HC diet (Fig. 2.5). Total apoB48 secretion was slightly higher for *Pemt*^{-/-} males under both dietary conditions, however these results were not significant. Thus, the decreases in total plasma apoB48 (Fig. 2.4B) observed in the male, HF/HC fed *Pemt*^{-/-} animals are probably due to increased catabolism. Control, chow fed animals demonstrated no changes in the secretion of apoB100 as predicted by total fasted plasma apoB (Fig. 2.4).

Figure 2.4: Separation and Analysis of Plasma ApoB48, ApoB100 and Apo A1.

The plasma of 2-3 animals was pooled to a total of 200 μ l and separated into various densities by ultracentrifugation. Lipoproteins were adsorbed by Cab-O-Sil extraction and Westerns were performed against the apoB containing lipoproteins (A-D) and apo A1 (E-F). Bands were quantitated using Image Gauge v3.0 software by Fuji and above results are expressed as the mean percentage of the largest band, $\pm$ S.E.M. Each analysis was done in triplicate and in each graph, $\square$ represents *Pemt*^{+/+} animals and $\blacklozenge$ represents *Pemt*^{-/-} mice. * $P < 0.05$ based on a Student's t-test.

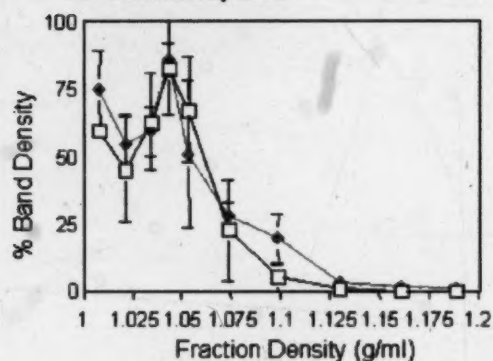
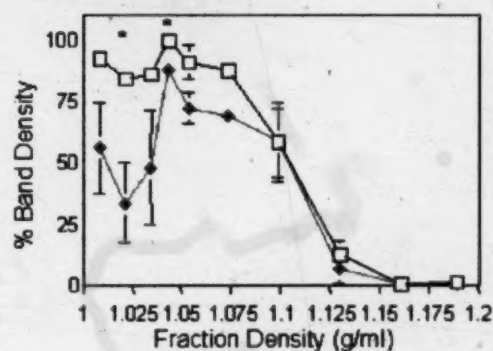
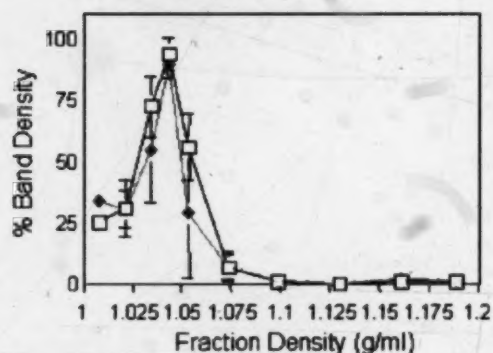
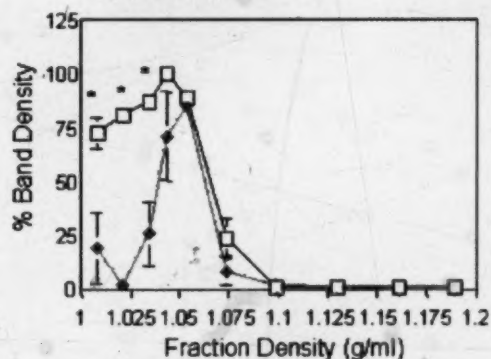
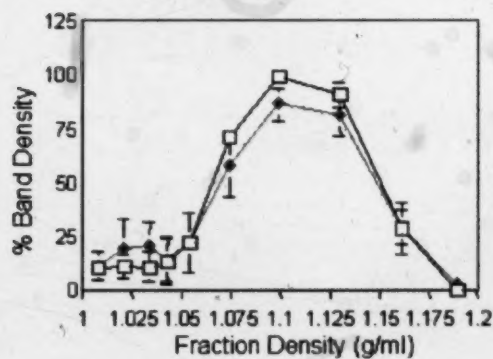
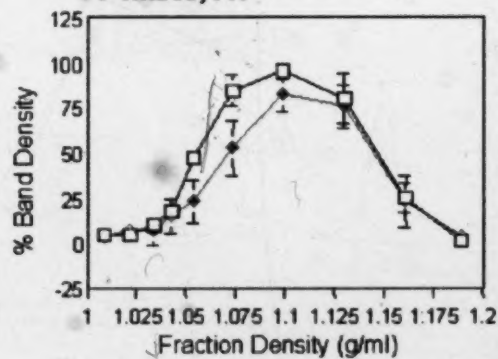
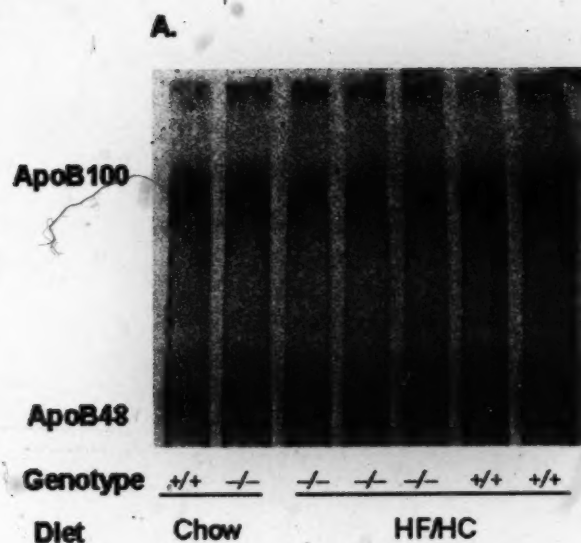
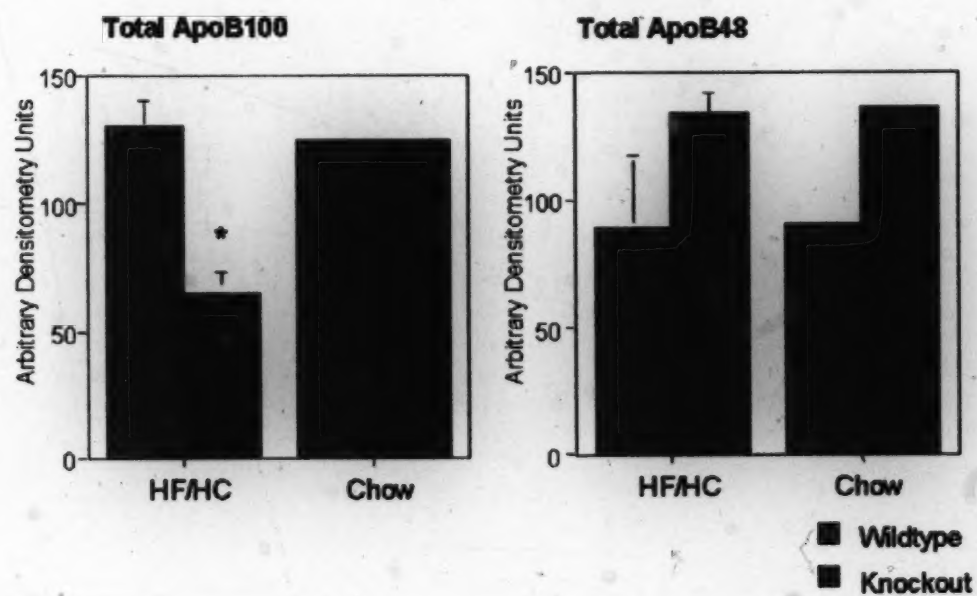
A. Females, B48**B. Males, B48****C. Females, B100****D. Males, B100****E. Females, A1****F. Males, A1**

Figure 2.5: Secretion of Total Plasma ApoB48 and ApoB100 in Male Mice

Male *Pemt*^{-/-} and ^{+/+} mice were put on a HF/HC or control diet for 3 weeks. At the end of the diets, the animals were fasted overnight and in the morning were injected with a total 200 μ l, 10% Triton WR 3119 (v/v) in PBS with 500 μ Ci [³⁵S]-Promix. The animals were sacrificed after 1 hr and apoB containing lipoproteins were immunoprecipitated from 100 μ l. A. A sample gel of the above experiment. B. Quantitation of apoB100 and apoB48 bands by Image Gauge v3.0 software. Above results are expressed as a mean of 4 animals for each genotype fed the HF/HC diets ($\pm$ S.E.M) and 2 animals of each genotype fed the chow diets. **P* < 0.05 based on a paired students t-test.



B.



2.4 Discussion

We have attempted to determine whether or not PEMT derived PC is targeted, or required for VLDL secretion. Previous lipoprotein analysis of choline supplemented, 8 week old *Pemt*⁺ mice showed normal secretion of all lipoproteins (12). Under the chow dietary conditions, this study also observed no differences in plasma VLDL in the mice. Therefore, PEMT derived PC is not required for VLDL secretion under normal dietary conditions.

However, we have now shown that PEMT contributes PC to lipoproteins in a gender specific, diet specific manner. In response to the HF/HC diet, the male *Pemt*⁺ mice were unable to increase plasma TG, and plasma VLDL as observed in the male ^{+/+} mice (Fig. 2.2B and 2.3). Concomitantly, TG levels accumulated in the livers of these male *Pemt*⁺ mice as shown by the lipid analysis (Table 2.2). Interestingly, although severe vacuolization was observed in the livers of the male *Pemt*⁺ mice, these did not stain with lipophilic stain as expected (Fig. 2.1D, F). The cause for this vacuolization is therefore unknown and requires further examination.

Analysis of apoB secretion in the male *Pemt*⁺ mice fed the HF/HC diet showed the decrease in plasma TG and VLDL to be specifically due to reduced secretion of apoB100 containing particles (Fig. 2.5). Secretion of apoB48 was unchanged in these animals even though fasted plasma apoB48 was also decreased (Fig. 2.4B). One possible explanation for this observation is increased catabolism of apoB48 containing particles, perhaps to compensate for the obvious decrease in apoB100 containing particles (Figs. 2.4 and 2.5). Why male HF/HC fed *Pemt*⁺ mice have defective VLDL secretion is not understood. Perhaps sufficient PC is available in these male mice under normal dietary conditions. Yet, with the HF/HC challenge there maybe insufficient PC for lipoprotein secretion, or perhaps sufficient PC is produced but it is not targeted to where it is required.

Although no significant changes were observed in plasma VLDL for the female *Pemt*⁺ mice, a 30-40% decrease in PC and cholesterol was observed in these animals under both dietary conditions. A large decrease in HDL derived PC and cholesterol was however observed consistently only in the HF/HC fed female *Pemt*⁺ mice (Table 2.4,

Figs. 2.2A and 2.3A, E) compared to their $^{+/+}$ counterparts. A 20% decrease in plasma PC and cholesterol was also observed in the male *Pemt* $^{-/-}$ mice fed a HF/HC diet. Difficulty was encountered in obtaining consistent HPLC profiles for some of the lipids (female plasma TG), especially results that correlated exactly with what was observed for total plasma lipid mass. It is important to point out that for each HPLC analysis, the plasma of only some of the animals was pooled whereas the results obtained for total plasma lipids were the average of all the animals analyzed. The fact that the animals are still in a mixed background, and the possibility of genetic drift was most likely the reason why differences in the lipoprotein profiles were encountered.

Previous studies in rats have shown that females rely more heavily on PEMT derived PC than males (14, 15). If this were the case in mice as well, perhaps the decrease in plasma PC in the female mice observed under the chow dietary condition results from some form of compensation for the loss of hepatic PEMT activity. It is unknown whether or not the *Pemt* $^{-/-}$ mice are taking up more HDL derived lipids to maintain appropriate hepatic PC levels, or they are secreting less PC and cholesterol into plasma HDL. The contribution of lipid to the HDL pool by the liver has not yet been elucidated. Nevertheless, maybe a compensatory mechanism is already established in the animals under chow conditions, thus the female *Pemt* $^{-/-}$ mice are able to tolerate the HF/HC diet and maintain normal plasma VLDL levels compared to the male *Pemt* $^{-/-}$ animals.

It is well understood that premenopausal women are less susceptible to cardiovascular disease generally due to low plasma LDL/HDL ratios (33). The cause of this protective effect in women is not well understood. However, finding differences in the regulation of VLDL secretion between the genders as shown in this study may lead to a better understanding of this mechanism. We suggest from these studies that some aspects of phospholipid metabolism differ between the genders, and this has an overall effect on the levels of plasma lipoproteins. Further studies need to be done to determine the mechanism(s) for this difference.

Interestingly, neither cellular (Table 2.2), nor biliary PC (Table 2.3) was significantly affected in any of the animals under any of the dietary conditions. It is understandable that cellular membrane integrity is of higher priority for PC distribution

than lipoprotein secretion. Because of the impact on membrane fluidity and integrity, membrane phospholipid concentrations are tightly regulated (35, 36). Obviously, this tight regulation applies even with the loss of a phospholipid synthetic pathway as observed in the *Pemt*^{-/-} mouse (12). However, why does the liver prioritize PC for bile over PC for VLDL secretion, primarily in the male *Pemt*^{-/-} mice? Maintenance of normal bile secretion is extremely demanding on PC levels since estimates suggest PC amounts equivalent to total hepatic PC, are secreted by the mouse into bile each day (13). We hypothesize that energy intake is of more importance here than hepatic lipoprotein secretion. Bile is required for the normal digestion and uptake of dietary fat, a very important source of energy for the body. Physiological design here is perchance directed more for times of starvation rather than times of dietary excess.

Because there were no specific inhibitors to PEMT activity, prior to the development of the *Pemt*^{-/-} mouse, it was difficult to study what purpose PEMT derived PC had in the liver. This was especially challenging since phospholipid metabolism is tightly regulated with usually more than one pathway for the production of any particular phospholipid (37). With the development of the *Pemt*^{-/-} mouse it was hoped that the role of this enzyme would be determined. This and other studies with this mouse show that the PEMT pathway has survived evolutionary selection for a number of complex functions that even now are not yet clear. However, in depth studies using the *Pemt*^{-/-} mouse will further help our understanding of phospholipid metabolism and homeostasis.

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Chapter 3

Insights into Gender and Dietary Regulation of Phosphatidylcholine Synthesis and Targeting in Mice

3.1 Introduction

PC is the primary phospholipid of eukaryotic cellular membranes playing a crucial role in structural maintenance of the lipid bilayer (1). In mammals PC also is the predominant phospholipid in bile, lung surfactant, plasma lipoproteins, and it plays a critical role as a second messenger in signal transduction (2). In all mammalian tissues, PC is made via the Kennedy pathway. This pathway relies on the intake of dietary choline, which is taken up by the cell and phosphorylated by CK. Phosphocholine is converted to CDP-choline by CT, followed by exchange of the CMP for a DAG group catalyzed by CPT (Fig. 1.10) (3).

In addition to the Kennedy pathway, PEMT is a liver specific, alternative route for PC synthesis (4). PEMT is a 22.3 kDa, transmembrane protein that catalyzes 3 consecutive methylations of PE to convert it to PC (5). PE methylation is the more ancient of the two PC synthetic pathways since it is the more predominant pathway in unique bacteria that synthesize PC (4,6), as well as in yeast (both pathways do exist in yeast) (7). Choline is readily available in mammalian diets, so it was not well understood why PEMT activity has survived evolutionary selection, and why its expression is limited to the liver.

The recent development of the *Pemt*^{-/-} mouse suggests that PEMT plays a critical role during times of choline deficiency such as starvation (8, 9). *Pemt*^{-/-} mice put on a CD diet displayed a severe decrease in PC synthesis and died after 3 days (9). In addition, studies described in Chapter 2 indicate that PEMT contributes to plasma lipoprotein levels in a gender and diet specific fashion. Male *Pemt*^{-/-} mice had decreased VLDL only when fed a HF/HC diet. Female *Pemt*^{-/-} mice displayed no changes in plasma TG or VLDL, however plasma PC and cholesterol was decreased in these animals, 30-40% in both chow and HF/HC fed animals (HDL fraction). These results suggest that in mice, PC metabolism and targeting differ between the sexes (10).

A number of previous studies have shown that PC metabolism differs in rats in a gender specific manner. Radiolabeling studies have suggested females rely more heavily on PEMT activity for PC synthesis than male rats (10). This was shown to have an effect on the fatty acid composition of hepatic PC in the female rats, specifically a higher

proportion of PC contained stearic and arachidonic acids compared to the male rats (11). It has been previously demonstrated in hepatocytes, and a hepatoma cell line, that PC species synthesized via methylation are primarily comprised of long chain, polyunsaturated fatty acids (12-14). Additionally, male rats are also more sensitive to CD diets with increased TG accumulation in the liver and decreased secretion of TG into the plasma compared to female rats (15). Nevertheless, the females could be reverted to a pattern similar to the males if given excess choline prior to the choline deficiency experiment (15). Also, female rat hepatic PC levels have been shown to be lower than males, causing a difference in the morphological appearance of the ER (16). Excess choline administration induced changes in the PC levels, as well as the ER, such that they too resembled observations made in male rats. Finally, it has also been determined that female Fischer-344 rats are resistant to choline deficiency induced hepatocarcinoma as compared to male rats (17).

In addition to gender specific regulation, PC metabolism is also regulated in response to various lipids. The rate limiting enzyme for the Kennedy pathway is CT and there are 3 isoforms: α (18), β_1 (19), and β_2 (20). All three isoforms are soluble in their inactive form and membrane bound when activated (primarily at the ER) (20, 21). Most studies have been performed on CT α , which unlike the other 2 isoforms is also localized to the nucleus and nuclear membrane (22). It has been established in hepatocytes that CT activity is mobilized to the active fraction in response to free fatty acids, while PEMT activity is inhibited under these conditions (23). More recent studies have shown that CT α is regulated transcriptionally (24) and post-transcriptionally (25) by the effects of cholesterol. Studies in alveolar type II epithelial cells verified that CT α expression was also stimulated by lipoprotein deprivation (26). Still, little is known about the regulation of PEMT activity and expression. In a number of studies, it has been demonstrated that PEMT and CT activities respond inversely to each other, and in this way these two pathways are regulated by some form of a feedback mechanism (8, 23, 27, 28).

With the gender and diet specific differences observed in our diet studies, we were curious to determine what changes in PC metabolism were occurring. In these studies, we examine the activities of both PEMT and CT in mice and show that in male mice, more CT activity is found in the active fraction compared to females. Furthermore,

in vivo radiolabeling studies show that the lipoprotein distribution of secreted PC differs in a gender and diet specific manner.

3.2 Materials and Methods

3.2.1 Materials: The semi-purified diet lacking a fat source was obtained from ICN. Silica gel G60 plates for TLC were from Merck. LPC and SM standards were purchased from Sigma. The TLC standard for PC, as well as the PMME substrate for PEMT assays were attained from Avanti Polar Lipids. The [^3H]-AdoMet, [^3H]-Met and [^3H]-choline radiolabels were purchased from Amersham-Pharmacia. Goat anti-rabbit conjugated horseradish peroxidase secondary antibody and the enhanced chemiluminescence detection system were from Pierce. Finally, cold AdoMet and Triton WR 1339 were purchased from Sigma. All other chemicals and reagents were purchased from standard commercial sources.

3.2.2. Care and Feeding of Mice: The *Pemt*^{-/-} mouse colony had a mixed genetic background of 129/J and C57BL/6 and was maintained via homozygous breeding in a reversed 12 hr-light/dark cycle (8). At the age of 12-14 weeks the animals were fed *ad libitum* either a control diet of regular rodent chow (PICO Lab Rodent Diet 20), or a HF/HC diet (29). This diet was made as described in Chapter 2 (29). Following 3 weeks, the animals were fasted overnight, and sacrificed.

3.2.3. Liver Processing and Protein Determination: Following extirpation, livers were rinsed with PBS and then homogenized in a glass-Teflon homogenizer in 3 ml of buffer (50 mM Tris-HCl, pH=7.5, 150 mM NaCl, 1 mM DTT, 0.1 mM PMSF, and 0.025% sodium azide) followed by sonication for 20 sec. Protein concentration was determined using the Coomassie Plus protein protocol from Bio-Rad, which is based on the Bradford assay (30). BSA was used as a standard for all protein assays.

For some of the enzymatic assays, membranes were isolated from the liver homogenates as follows. Total homogenate was first centrifuged at 600 g for 10 min to pellet unbroken cells and nuclei and the supernatant was centrifuged at 100 000 g for 1 h.

The membrane pellets were resuspended in the above buffer and homogenized using a glass-Teflon homogenizer. The supernatant constituted the soluble, inactive fraction.

3.2.4. PEMT and CT Enzymatic Assays: Specific *in vitro* PEMT activity was measured from total liver homogenate as previously described (31). CT activity measurements were performed for total liver homogenate, soluble and membrane bound fractions in the presence of PC/oleate vesicles as previously described (32).

3.2.5. PEMT Westerns: Total homogenate dilutions (1mg/ml) were boiled for 5 min in Laemmli buffer (33) and proteins were resolved on a 12% SDS-PAGE gel (35 μ g protein for each well). Proteins were transferred to nitrocellulose membranes and probed with the anti-PEMT2 (5) antibody followed by a goat-antirabbit horseradish peroxidase conjugated secondary antibody. Bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions.

3.2.6. *In vivo* Radiolabeling of PEMT Derived PC: Male and female *Pemt*^{+/+} mice were put on the HF/HC or chow diets for 3 weeks as described (Chapter 2). Following the 3 weeks, the animals were fasted overnight and in the morning were injected with 200 μ l PBS containing a total 10% Triton WR 1339 (v/v) and 100 μ Ci [³H]-Met (5 mCi/ml). The animals were harvested after 5 hr and blood, liver and bile were collected.

Blood was collected via the lower *vena cava* in the presence of trace amounts of 250 mM EDTA, and plasma was isolated by centrifugation. Then, 2 separate 200 μ l samples of plasma for each animal were separated into 10 density fractions each. Briefly, each 200 μ l sample was mixed with 1.3 ml, 4.151 M KBr and loaded at the bottom of a 5 ml Quick-Seal tube. Each sample was overlaid with 0.9% NaCl and spun at 416 000 g for 1 hr in a Beckman Vti 65.2 rotor (34). Lipid extraction on each set of samples was performed using a modified Bligh-Dyer protocol (35). The phospholipids, from each set of density fractions, were separated by TLC using a developing solvent of chloroform/methanol/acetic acid/water (25:15:4:2). Lipid bands were visualized with iodine vapour and the bands of interest were scraped. One set of samples for each animal

was analyzed for phospholipid mass using the Malachite Green lipid phosphorus assay (36). The radioactivity in the phospholipids of the second set of density fractions was measured by liquid scintillation counting in order to analyze the PEMT derived PC. Total plasma phospholipid mass and radiolabel were determined by adding the values obtained from the 10 density fractions.

Lipids were extracted from 8 μ l of bile and the phospholipids were separated by TLC as described above. Two sets of TLC plates were resolved for each bile sample with half of each sample measured for radiolabeled phospholipids, and the other half for phospholipid mass. Livers were homogenized as described above and protein concentration determined. Total homogenates were diluted to 1 mg protein/ml and lipids were extracted and separated via TLC as described above. Again, both incorporation of radiolabel and phospholipid mass were determined for the livers as described for the plasma samples.

3.3 Results

3.3.1 Characterization of PEMT Activity and Distribution in the Mice:

We first determined whether or not PEMT activity or protein mass was regulated in a gender or diet specific fashion. *Pemt*^{+/+} mice were fed for 3 weeks, chow or HF/HC diets, followed by an overnight fast and sacrifice. *In vitro* PEMT assays were performed on the livers of these animals and it was demonstrated that the levels of specific PEMT activity did not differ in response to either the diet, or gender (Fig. 3.1A).

In rats it is well established that PEMT is distributed between two cellular fractions, the ER and the MAM (5). A peptide antibody raised against the C-terminal 12 residues of the rat PEMT protein only cross reacts against the PEMT protein localized in the MAM fraction and not in the ER fraction (5). Ironically, the majority of PEMT activity is located in the ER. The cause for the variance at the C-terminus of the PEMT protein has not been elucidated. Furthermore, the function of 2 separate PEMT activities at the 2 different cellular fractions has also not been established.

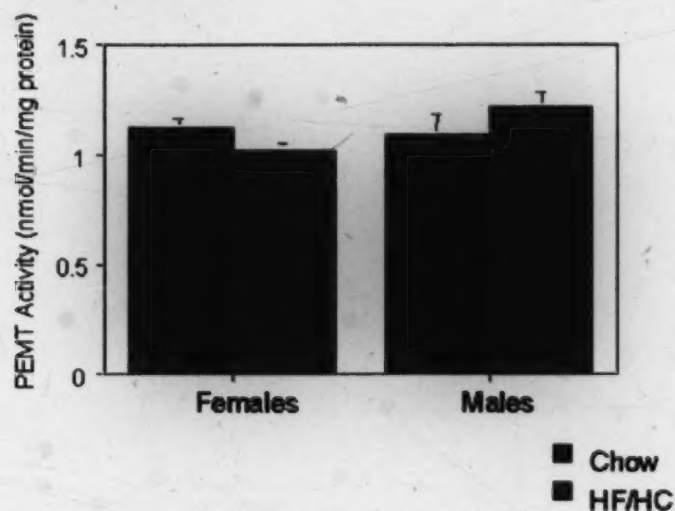
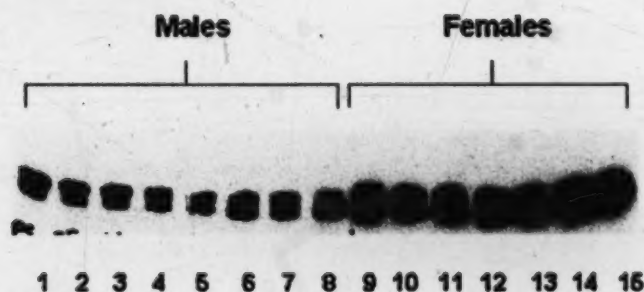
A. Total PEMT Activity**B. PEMT Western**

Figure 3.1: PEMT Activity and Protein Expression in Mice as a Function of Diet and Gender.

Twelve to 16 week old *Pemt*^{+/+} animals were fed either a HF/HC or a chow diet for 3 weeks. The animals were fasted overnight and sacrificed. (A) Total liver homogenates were assayed for PEMT activity. Shown are the mean values $\pm$ S.E.M, $n=4-5$. (B) Immunoblots against total liver homogenates of female and male *Pemt*^{+/+} on both diets using the anti-PEMT2 antibody specific for the C-terminus of the protein. Each lane contained 35 μ g of protein. Lanes 1, 3, 5, 7, 12, 13 and 15 are samples from chow fed animals. Lanes 2, 4, 6, 8, 9-11 and 14 are from HF/HC fed animals

Western blots were performed against total liver homogenates of female and male *Pemt*^{+/+} animals fed the two diets, using the antibody raised against the PEMT C-terminus. We were surprised to observe that the antibody cross-reacted with the female samples twice as strongly as the males (Fig. 3.1B). This result provides preliminary evidence suggesting that the PEMT protein is distributed in the cell in a gender specific fashion. In the females, more protein is located in the MAM fraction as compared to males. Still, the lack of change in *in vitro* PEMT activity suggests that PEMT protein mass is unchanged.

3.3.2. CT Activity is Distributed in a Gender and Diet Specific Fashion:

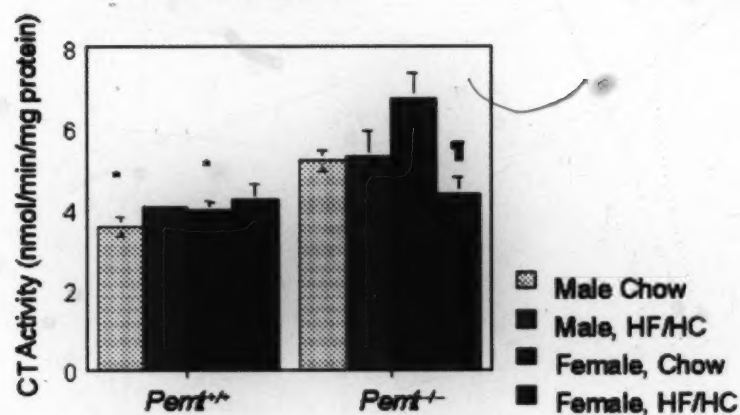
Next, we were interested in understanding why the Kennedy pathway is unable to compensate for the loss of PEMT activity in the male *Pemt*^{-/-} mice fed a HF/HC diet (Chapter 2). Specific CT activity was increased in male and female *Pemt*^{-/-} mice fed a chow diet compared to the *Pemt*^{+/+} animals (Fig 3.2A). These results suggest that these animals are trying to compensate for the loss of PEMT activity by upregulating total CT activity under normal dietary conditions. Interestingly, there was no upregulation in the *Pemt*^{-/-} animals fed a HF/HC diet compared to the ^{+/+} animals. In fact, a significant loss in total activity in response to the HF/HC diet was measured in the female *Pemt*^{-/-} animals compared to their chow fed counterparts (Fig. 3.2A).

Under all dietary and gender conditions, the *Pemt*^{-/-} animals had significantly more CT located at the membranes (Fig. 3.2B). This result concurs with previous observations (8) and again suggests that the *Pemt*^{-/-} mice must compensate for the loss of PEMT activity by upregulating PC production via the Kennedy pathway. Surprisingly, in all genotypic and dietary conditions, male mice had 1.5-3 fold higher membrane bound CT specific activity compared to females (Fig. 3.2B). This result suggests that male mice are more dependent on the Kennedy pathway than females since a higher percentage of their total CT protein is located in the active fraction. Female *Pemt*^{+/+} animals fed the HF/HC diet had significantly higher level of CT activity in the cytosol compared to female *Pemt*^{+/+} animals fed a chow diet. Otherwise, there were no significant trends in the cytosolic CT activities between *Pemt*^{-/-} mice and their ^{+/+} counterparts as a result of gender or diet.

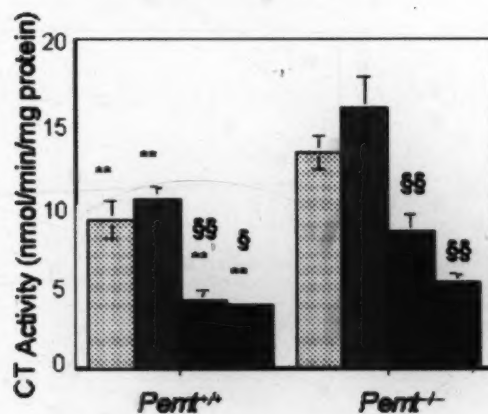
Figure 3.2: CT Activity is Regulated by Genotype and Gender.

(A) Total liver homogenates from *Pemt*^{+/+} and ^{-/-} animals on the 2 diets were assayed for CT activity in the presence of PC/oleate vesicles. Values are expressed as the mean $\pm$ S.E.M, and 3-5 animals were assayed for each condition. Based on a Student's t-test, * $P < 0.01$ where *Pemt*^{+/+} animals are compared to *Pemt*^{-/-} animals of identical gender and diet. ¶ $P < 0.05$ where *Pemt*^{-/-} chow and HF/HC females are compared. (B) Membranes and cytosol were separated by ultracentrifugation. CT assays were performed on the membranes and data is expressed as the mean $\pm$ S.E.M. ** $P < 0.05$ represents Student t-tests comparing *Pemt*^{+/+} animals to their ^{-/-} counterparts. § $P < 0.01$ and §§ $P < 0.05$ represent Student t-test comparisons of female mice to their male counterparts. (C) CT assays performed with the cytosol. Cytosolic CT activity increased significantly in the HF/HC fed female *Pemt*^{+/+} animals as compared to the chow fed mice (¶ $P < 0.05$).

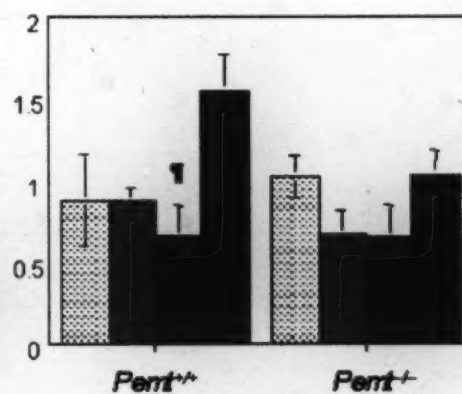
A. Total Homogenate



B. Membrane Bound



C. Soluble



3.3.4. *In vivo* [^3H]-Methionine Labeling in the Presence of Triton WR 3119:

Since both the Kennedy and PEMT pathways for PC metabolism differ in response to either gender or diet (Fig. 3.1, 3.2), we were interested in the *in vivo* synthesis and targeting of PEMT derived PC in these animals. In our diet study, we observed unique gender and diet specific effects on apoB100 containing lipoproteins in response to the loss of PEMT activity (Chapter 2). Thus, we were specifically interested in determining whether or not PEMT derived PC is specifically targeted for VLDL secretion. Since VLDL is rapidly catabolized in the mouse we chose to label the PEMT derived phospholipid *in vivo* with [^3H]-Met in the presence of Triton WR 3119. Triton WR 3119 is a detergent used commonly to study the rate of VLDL secretion since it inhibits LPL (37).

Since this type of method has not been performed with regards to phospholipid metabolism, a control study was performed to confirm that VLDL PC was intact under these circumstances. Four adult male C57BL/6 mice fed regular chow were fasted overnight and then injected with 200 μl PBS containing 100 μCi [^3H]-Met in the presence or absence of 10% (v/v) Triton WR 3119 (38). The animals were sacrificed 2.5 and 5 hr post-injection and the plasma was separated into density fractions by ultracentrifugation. Measurements were made for all the choline derived phospholipids. From this pilot study it was clear that the Triton WR 3119 inhibited the catabolism of the VLDL particles, since the radiolabeled PEMT derived PC remained in the lower density fractions (Fig. 3.3). In the animals injected with PBS alone, radiolabeled PC in the VLDL fraction essentially disappeared and shifted to the higher densities. The 5 hr time point was chosen for our experiments since the radiolabel found in all the choline-derived phospholipids (data for LPC and SM not shown) was optimal at this time point.

3.3.5. PEMT Derived PC is Targeted Differently in Response to Diet and

Gender in the Liver: *Pemt*^{+/+} females and males were put on 3 week chow or HF/HC diets and fasted the night prior to the *in vivo* labeling procedure. Each animal was injected with [^3H]-Met and Triton WR 3119 as described above and sacrificed 5 hr

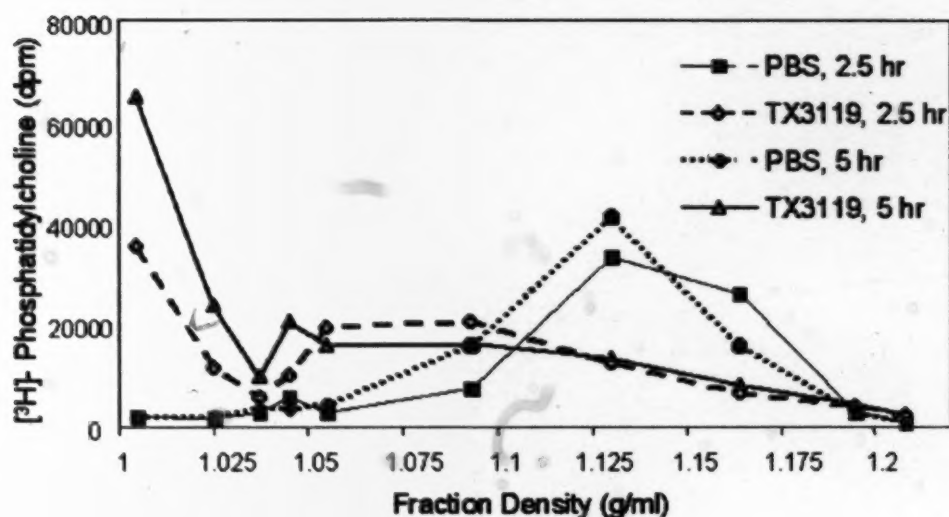


Figure 3.3: Pilot Study to Examine the Effectiveness of Triton WR3119 Maintenance of VLDL PC.

Four adult male C57BL/6 mice were fasted overnight and injected with 200 μ l total volume of PBS, with or without 10% (v/v) Triton WR 3119. [^{3} H]-Met (100 μ Ci) was also injected to radiolabel PEMT derived PC. Animals were sacrificed after 2.5 and 5 hr and plasma was separated into various densities. Lipids were extracted, resolved via TLC and PC, SM and LPC were scraped and measured by scintillation counting. Shown are results obtained from the PC measurements.

post-injection. Liver, bile and plasma were isolated from all the animals and processed as described in section 3.2.6.

The livers were examined for any differences between the groups. No significant trends were observed with regards to total PC, LPC or SM mass in the livers in any of these animals (Fig. 3.4A). However, the levels of radiolabeled PC were higher in the chow fed animals compared to the HF/HC fed animals (Fig. 3.4B). This trend was observed for both males and females but was significant only in the females due to large variations in the numbers attained from the male, chow fed animals. These results

suggest that at least in female mice, the level of fat and cholesterol in the diet has an effect on the rate of PEMT derived PC production or removal from the liver.

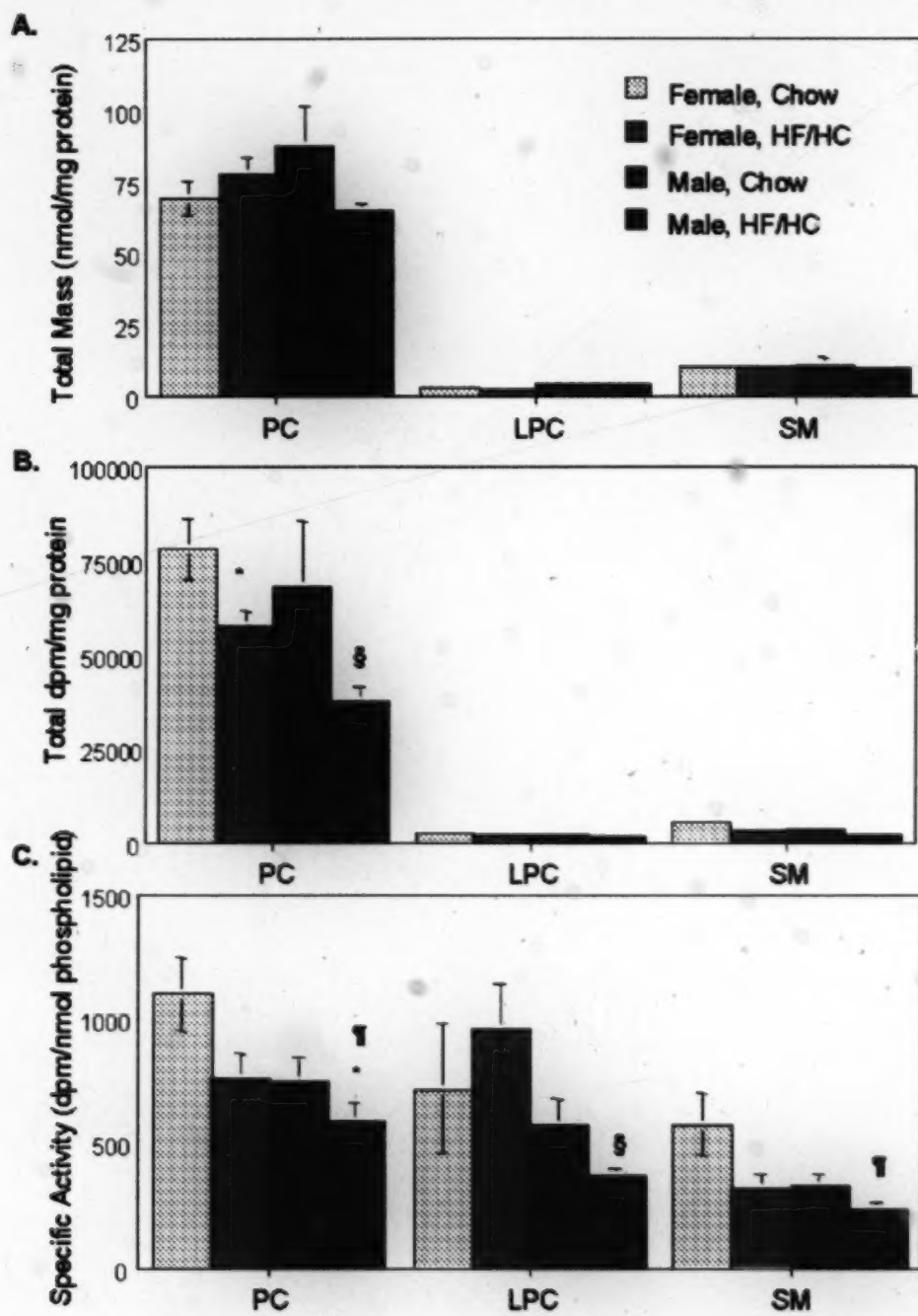
Interestingly, even though a significant decrease in [^3H]-Met labeled PC was observed in the female mice fed a HF/HC diet, this did not translate into a significant change in specific activity (Fig. 3.4C). Levels of PEMT derived PC were the lowest in the male HF/HC fed animals (Fig. 3.4B) resulting in a significantly decreased specific activity compared to the chow fed males (Fig. 3.4C). Overall these results suggest that PC metabolism in the liver is affected by diet. Radiolabeled PEMT derived PC was generally decreased in both male and female mice fed the HF/HC diets. Small, but insignificant changes in total PC mass in the livers of these animals affected the specific activity of the PEMT derived PC suggesting that the Kennedy pathway is also potentially affected.

Gender also contributed to the differences in levels of PEMT derived PC in the livers of these animals. Levels of [^3H]-Met labeled PC were significantly higher in female HF/HC fed animals compared to the males fed the same diet (Fig. 3.4B). This suggests that in the HF/HC fed female mice, either PEMT produces PC more rapidly or this PC pool is not depleted as quickly. Overall, the trends suggest that [^3H]-Met labeled PC accumulated more quickly in the females compared to males, and in chow fed animals over HF/HC fed animals. As a result of these trends, the specific activity in the livers of male HF/HC fed animals was significantly lower compared to the chow fed female mice (Fig. 3.4C).

Radiolabeled SM and LPC were also analyzed to determine whether or not PEMT derived PC is targeted for conversion to either of these phospholipids in the hepatic tissue. The trends with regards to specific activity for SM were similar to PC. Decreases were observed in response to gender and diet but again, this trend was only significant when female chow fed mice were compared to male HF/HC fed animals (Fig. 3.4C). Specific activity for radiolabeled LPC was regularly higher in the female livers compared to the males, for the HF/HC fed animals (Fig. 3.4C).

Figure 3.4: PEMT Derived Phospholipid Levels Differ in the Liver in Response to Gender and Diet.

Female and male *Pemt*^{+/+} mice were fed either HF/HC or chow diets for 3 weeks. Animals were fasted overnight and then injected with PBS, Triton WR 3119 and [³H]-Met. Animals were sacrificed after 5 hr, the livers were homogenized, lipid extracted and TLC performed. (A) Phospholipid mass was determined using a Malachite Green lipid phosphorus assay. (B) PEMT derived PC, LPC and SM as determined by scintillation counting. The data is expressed as the mean of values, $\pm$ S.E.M. * $P < 0.05$ from a Student's t-test comparing the effect of diet within the genders. § $P < 0.01$ compares the female and males fed the HF/HC diet. (C) Specific activity was determined by dividing the radiolabel values by the total mass of phospholipid. * $P < 0.05$ represents comparison of the effect of diet within the gender. § $P < 0.05$ represents the effect of gender, and not diet, on the specific activity of LPC in the livers. ¶ $P < 0.05$ where the combined effect of gender and diet is examined by a Student's t-test (female chow values compared to male HF/HC fed animals).



3.3.6. PC is Targeted Differently to Bile in Response to Diet and Gender:

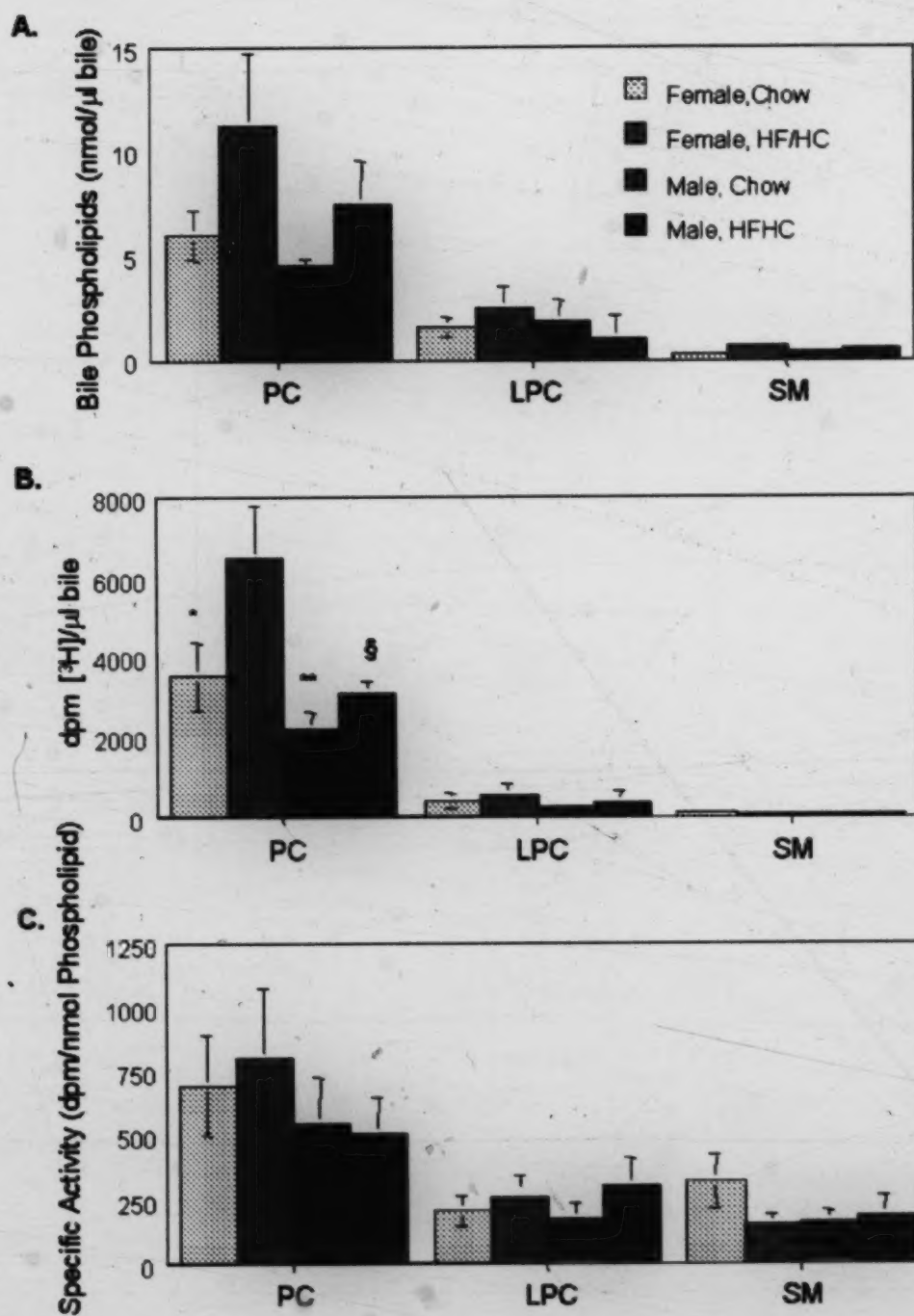
With the increased demand for bile in the HF/HC fed animals, total PC secretion into bile increased for both males and females compared to the chow (Fig. 3.5A). These changes were not significant however, because of high animal variability. The trends for PEMT derived PC, as shown by the radiolabel, mimicked the trends observed for total PC mass (Fig. 3.5B). The increase in the HF/HC fed males as compared to their chow fed counterparts was significant ($P=0.0267$), while in the females the increases approached significance ($P=0.051$) (Fig. 3.5B). Because the trends for both total PC mass and PEMT derived PC resembled each other there was no change in specific activity in response to diet (Fig. 3.5C). Overall, these results suggest that an increase in fat and cholesterol in the diet stimulates PC secretion into the bile. PEMT derived PC contributes to this pool of PC, but it is not specifically targeted for bile secretion. The trends observed for radiolabeled PC targeted for bile were the inverse of findings made in the liver (Fig. 3.4). In response to the HF/HC diet, PEMT derived PC levels diminished in the liver and increased in the bile suggesting that diet can significantly alter PC demand on the liver.

Minor differences were also observed for PEMT derived PC targeted to bile because of gender. As observed in the liver samples, levels of [^3H]-Met labeled PC were generally higher in the females compared to the males (Fig. 3.5B). These changes approached significance for the HF/HC fed animals ($P=0.05$). Again, the trends observed for the radiolabeled PC mimicked data observed for PC mass, resulting in no change in specific activity as a result of gender.

PC is the predominant phospholipid secreted into bile, while SM and LPC are very minor components. It was still necessary however to confirm that PEMT derived PC was not targeted for some reason into these phospholipid pools. Levels of radiolabeled LPC and SM were very low and no significant observations were made in regards to diet, or sex.

Figure 3.5: Secretion of PEMT Derived PC into Bile Differs in Response to Both Diet and Gender.

(A) Total mass of phospholipids derived from bile as determined by Malachite Green assay. (B) PEMT derived phospholipids as determined by levels of [^3H]-Met labeling. Data is expressed as the mean of values, $\pm$ S.E.M. * $P = 0.051$ and ** $P < 0.05$ based on Student t-tests determining the significance of differences caused by diet within each gender. § $P = 0.05$ where gender is compared (specifically female and male animals fed a HF/HC diet). (C) Specific activity of PEMT derived phospholipids where radiolabel values were divided by total phospholipid mass. No significant changes or trends were observed.



3.3.7. PEMT Derived PC is Targeted Differently to Lipoproteins in Response to Diet and Gender:

The lipoprotein distribution of total and radiolabeled PC, LPC and SM was examined in all of the animals. In the male mice fed a HF/HC diet, data for both PC mass and for [^3H]-Met demonstrated a shift from the VLDL fraction (density: 1.009 g/ml) to the HDL fractions (densities 1.067-1.118 g/ml) (Fig. 3.6A,B). For chow and HF/HC fed females, as well as chow fed males, 45% of the total PC mass was in the VLDL fraction (Fig. 3.6A). In the male HF/HC fed mice, this value was diminished to 25%. Similar decreases in the percent distribution of [^3H] labeled PC was also observed in the VLDL fractions of these animals (Fig. 3.6B). Because the distribution of radiolabeled PC resembled the pattern for total PC, no changes in specific activity were observed in the lipoprotein distributions for any of the mice (data not shown).

Targeting of radiolabeled LPC and SM to the VLDL fraction was significantly higher in female, chow fed mice compared to all the other study groups (Fig. 3.7A, B). Targeting of [^3H]-Met labeled LPC to the HDL fractions was observed in the female chow fed animals and these values decreased with the HF/HC diet as well as in response to gender. Still, these trends were only significant when the female chow mice were compared to the male mice fed the HF/HC diet.

Total plasma radioactivity and phospholipid mass was calculated by adding the values for all 10 density fractions isolated from each mouse (Fig. 3.8). Total plasma PC levels increased steadily from the female chow fed animals to the male HF/HC fed animals (Fig. 3.8A). In this case, gender seemed to play a more important role in determining total plasma PC. Male chow fed mice had significantly higher PC levels than their female counterparts ($P=0.029$) while the increases in the HF/HC fed males compared to females approached significance ($P=0.056$). Again, the combined effect of diet and gender resulted in a significant increase in the male HF/HC fed animals over female chow fed animals with regards to plasma PC mass. No significant changes were observed with regards to targeting of PEMT derived PC to the plasma in response to either diet or gender (Fig. 3.8B).

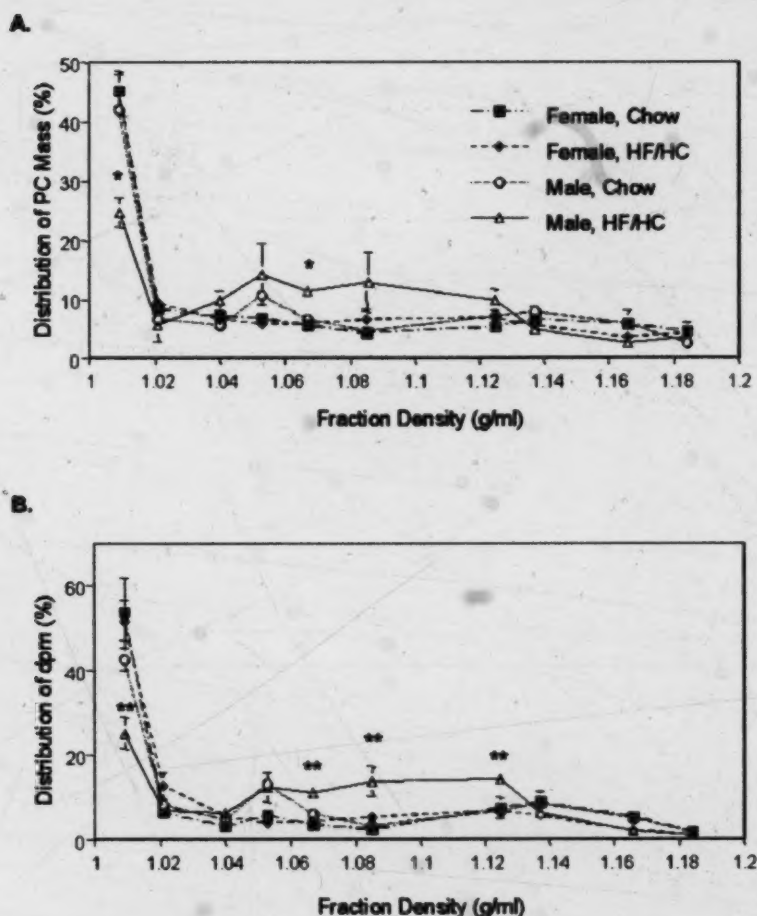


Figure 3.6: PEMT Derived PC is Distributed Differently in Male HF/HC Fed Mice.

Two 200 μ l samples of plasma from each animal injected with [^{3} H]-Met and Triton WR 3119, were separated into density fractions. One sample was used for total phospholipid mass measurements and the other for radiolabel measurements. (A) Distribution of PC mass was determined by calculating the percent of the total PC mass in each density fraction. Data is displayed as the mean of values, $\pm$ S.E.M. * $P < 0.01$ where the male HF/HC fed animals were compared to all the other study groups (ANOVA). (B) Distribution of [^{3} H]-PC was determined by calculating the percent of the total radiolabel each in density fraction. Values are expressed as mean percentage $\pm$ S.E.M. ** $P < 0.05$ where the male HF/HC fed animals were compared to all the other study groups via ANOVA. Densities represent the following fractions: 1.010g/ml, VLDL; 1.020-1.060, LDL; and 1.061-1.185 g/ml the HDL fraction.

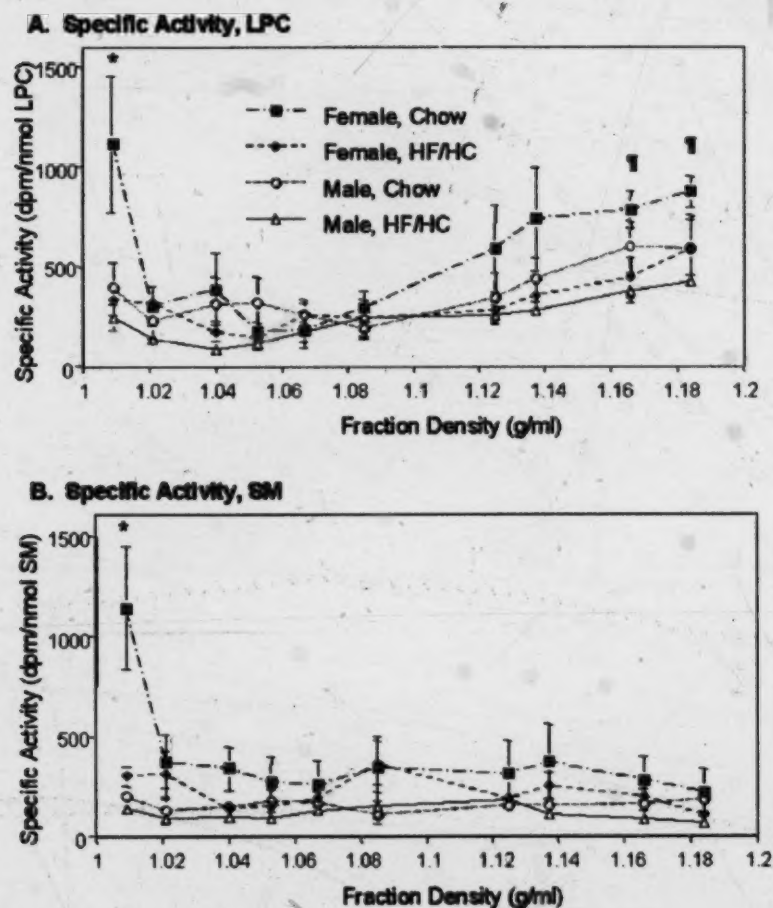
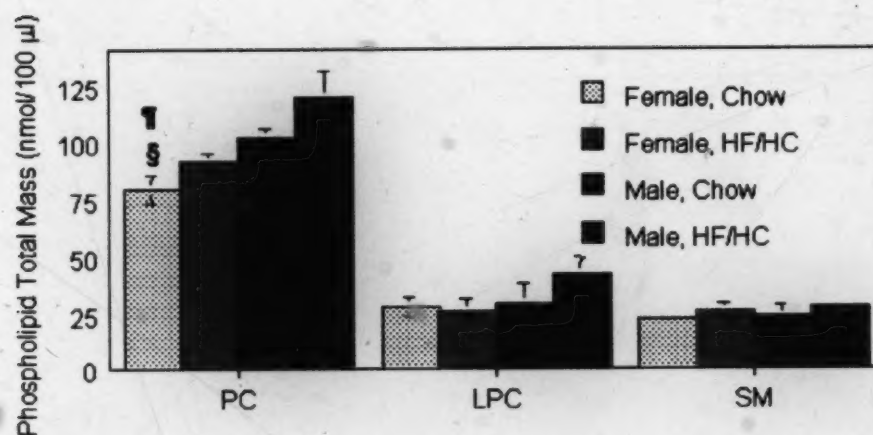
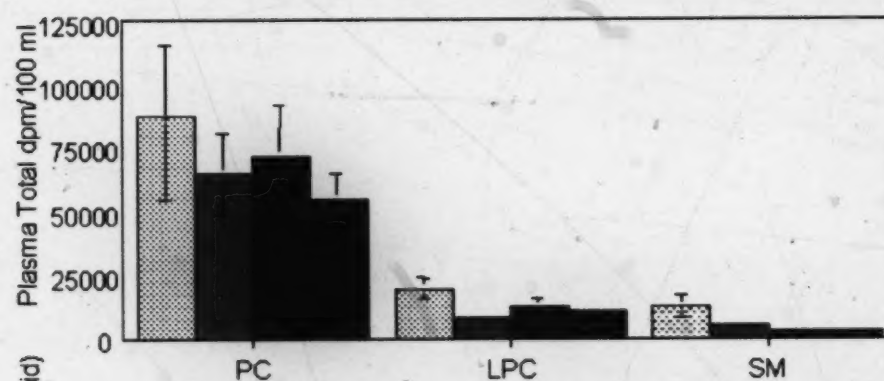
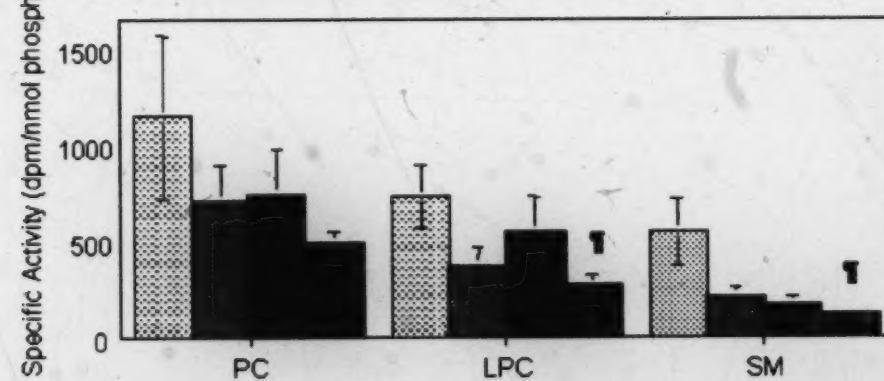


Figure 3.7: PEPT Derived LPC and SM are Targeted for VLDL Secretion in Female Chow Fed Animals.

The specific activity for each density fraction was determined by dividing the [^3H] dpm values by total phospholipid mass for LPC (A) and SM (B). Significant targeting of PEPT derived LPC and SM into the VLDL fraction was tested by comparing the female chow values to numbers attained from all other study groups (ANOVA, * $P < 0.05$). Overall, decreases in targeting of PEPT derived LPC into HDL fractions as a result of both diet and gender was also tested for significance. ¶ $P < 0.01$ as determined by a Student's t-test comparing female chow mice to male HF/HC fed animals.

Figure 3.8: Effects of Diet and Gender on Targeting of PEMT Derived Phospholipids to Plasma.

Total values for (A) phospholipid mass, (B) [^3H]-Met labeled phospholipids were determined by adding all 10 values from the density fractions. Specific activities (C) were determined by dividing the total radiolabel values by the total mass values for each mouse. (A) Total plasma PC mass increases predominantly in response to gender. $\S P < 0.05$ where female and male chow values are compared by a Student's t-test. The HF/HC diet also increases levels of plasma PC however not significantly within the gender groupings. However the combination of gender and diet resulted in significant difference in plasma PC mass between female chow and male HF/HC fed animals ($\P P < 0.05$). (B) No significant differences were observed in PEMT derived phospholipids in the plasma in response to diet. However the combination of trends in total mass and total radiolabel resulted in significant trends for specific activity. Female chow values compared to male HF/HC fed animals were significantly increased ($\P P < 0.05$).

A.**B.****C.**

Plasma SM and LPC masses were unchanged in response to any of the conditions. However, the female chow fed animals secreted both radiolabeled LPC and SM phospholipids most efficiently. This decreased with feeding a HF/HC diet, and also in the male mice. Again this trend resulted in a significant difference in the specific activities for both LPC and SM in male HF/HC fed animals compared to female chows (Fig. 3.8C).

3.4 Discussion

In this study we were interested in determining whether or not diet or gender play a role in regulating PC metabolism. This interest was prompted by results obtained in our diet study. VLDL secretion was impaired in male *Pemt*^{-/-} mice fed a HF/HC diet and as a result, the livers of these animals accumulated 2-fold TG compared to *Pemt*^{+/+} males fed the same diet. Since similar observations were attained when *Pemt*^{-/-} mice were fed a CD diet (9), we questioned whether or not PC production via the Kennedy pathway was sufficient in these male animals.

Initially, we measured PEMT activity and distribution in the livers of *Pemt*^{+/+} females and males fed either a chow or a HF/HC diet. Specific PEMT activity was identical in all the study groups (Fig. 3.1A). Nonetheless, Western blots using the anti-PEMT antibody raised against the C-terminus (5) suggested that in the cell, the PEMT protein is distributed differently in a gender specific manner (Fig. 3.1B). The antibody, which is known to only cross-react with PEMT activity associated with the MAM (an extension of the ER which is in close proximity to the mitochondria) (39), cross-reacted with the female samples with higher affinity than the males (Fig. 3.1B). These results imply that a higher percentage of the total PEMT protein is distributed to the MAM in the female mice compared to the males. Still, further experimentation such as PEMT activity assays on specific cell fractions are required to confirm these preliminary results.

The function of MAM is unknown, other than it has a high concentration of lipid producing enzymes and has been hypothesized to play a role in transport of lipids between the mitochondria and the ER (40). The reason why PEMT activity is localized at different cellular fractions is unknown, and the mechanism for targeting to the MAM versus the ER has not been elucidated. Our lab developed a method to separate these two

fractions using a DEAE-Sepharose column (A. Noga *et al.*, unpublished results). From this separation we know that the MAM associated PEMT protein is more positively charged than the ER localized PEMT. A number of potential phosphorylation sites exist in the PEMT sequence, one which is within the last 12 residues. Still it has not been demonstrated yet that PEMT is phosphorylated *in vivo* (41). Otherwise, there are no known consensus sites for other forms of post-translational modification in the PEMT sequence (D.E. Vance, unpublished results). As well, northern blots probing for murine PEMT mRNA, revealed only 1 band, implying that the change in MAM and ER localized PEMT is not likely due to alternative splicing (42).

The predominant pathway for PC synthesis in the liver is the *de novo* Kennedy pathway. In this study, we investigated the regulation of the rate-limiting enzyme, CT. From our analysis it was shown that hepatic specific CT activity was slightly elevated in chow fed *Pemt*^{-/-} animals as compared to *Pemt*^{+/+} mice (Fig. 3.2A). We hypothesize that these animals are trying to compensate for the loss of PEMT, an important contributor of PC in the liver. Surprisingly, this increase was lost in the HF/HC fed *Pemt*^{-/-} animals compared to the *Pemt*^{+/+} mice fed the same diet. This may be explained by inactivation of SREBP mediated transcription of the CT gene due to increased cholesterol (24). Nevertheless, the increased consumption of free fatty acids from the diet would be expected to increase the requirement for CT activity (25). Further investigation will be required to understand the combined role of cholesterol and fatty acids on CT regulation.

When the CT activity was separated to membranes and cytosol, we identified a 1.5-3 fold increase in membrane bound activity in male mice compared to the females (Fig. 3.2B). While it is unclear which CT isoform is responsible for this increase, we presume it to be CT α as it is the most highly expressed isoform found in hepatic tissue (20). Trends with regards to cytosolic CT activity were not so clear (Fig. 2C). The difficulty to interpret the cytosolic results may stem from the additional site of the CT α isoform to the nucleus as well as the nuclear membrane (22). A previous study has shown that the CT α protein shuttles between the nucleus, ER and cytosol (21). Unbroken nuclei are lost in our processing of the livers to soluble and insoluble fractions and so there may have been a considerable loss of this major CT activity in our studies.

Difficulty in interpreting results from the inactive fraction has also been encountered in other studies (25).

Diet seemed to play a lesser role with regards to localization of CT to the active fraction. Although some trends were observed, in most cases the differences were not significant. Both CT α (43) and CT β_2 (20) contain a number of phosphorylation sites at their C-termini that are hypothesized to play a regulatory role in the activation/inactivation of these enzymes. It would be interesting to determine whether or not these sites are playing an integral role in the gender specific, diet specific regulation of CT activity in these animals.

From the above results it is clear that gender is playing a role in the regulation of PC metabolism. Based on the separation of CT activity into membrane bound and soluble fractions, it appears that male mice have more CT protein activated compared to females. These results in mice comply with previous studies performed in rat, which verified that male rats rely more heavily on the Kennedy pathway for their hepatic PC than females (10,11,15,16). To understand what role gender and diet were playing *in vivo*, we radiolabeled PEMT derived PC with [^3H]-Met in *Pemt*^{+/+} males and females on either chow or HF/HC diets. This experiment was novel in that we also injected these animals with the radiolabel in the presence of Triton WR 3119 so that we could specifically examine the targeting of PEMT derived phospholipids into VLDL.

In response to the HF/HC diet, liver total phospholipid remained normal for both the females and the males (Fig. 3.4A). However, this diet diminished the amount of radiolabeled PC in the livers compared to chow fed animals (Fig. 3.4B). Furthermore, the females seemed to have higher levels of PEMT derived PC in their livers compared to the males (Fig. 3.4B). Overall, the trends suggested that both the male gender, as well as the HF/HC diet decreased levels of PEMT derived PC in the livers. Differences within the dietary groups or genders were not necessarily significant however the steady decrease observed from female chow fed animals down to male HF/HC fed animals was significant for both total radiolabel as well as specific activity (Fig. 4B,C). Similar gender and dietary trends affected the specific activity for SM. Only a gender specific effect was observed in the specific activity of radiolabeled LPC (Fig.3.4C).

The loss of PEMT derived PC observed in the hepatic tissue in response to the HF/HC diet was recovered in the bile. Significant increases in radiolabeled PC were observed in both female and male mice fed this diet compared to the chow fed animals (Fig. 3.5B). The HF/HC fed male mice also had decreased levels of biliary [^3H]-PC compared to the females. Although these changes were observed in the bile with regards to PEMT derived PC, no changes were found for specific activity (Fig. 5C) because the trends observed for the radiolabeled PC, mimicked observations made for PC mass (Fig. 3.5A). In summary, it appears that the HF/HC diet increases the demand on PC for bile secretion, resulting in movement of PEMT derived PC from the liver to the bile. Still, because virtually no change in specific activity was observed, it appears that PEMT derived PC is not specifically targeted for bile secretion.

Lipoprotein separation of both total PC as well as PEMT derived PC confirmed that distribution of this phospholipid could be influenced by a combination of gender and diet. Significant decreases for both total PC as well as [^3H]-PC in the VLDL fraction for male HF/HC fed animals was observed as compared to all the other gender and dietary groups (Fig 3.6). This decrease in VLDL PC was recovered in the HDL density fractions. These results were surprising in lieu of results observed in Chapter 2. In the *Pemt*^{-/-} males fed a HF/HC diet VLDL secretion was impaired. These results suggested that male mice rely heavily on PEMT derived PC for VLDL secretion when challenged with a HF/HC diet. Here we observed that not only is PEMT derived PC not targeted for VLDL secretion under these conditions, but also total PC mass was depleted in this fraction.

The mechanism for this change in distribution is unknown and requires further study. Perhaps male mice rely more on smaller apoB48 containing particles for secretion of neutral lipid from the liver under the HF/HC fed conditions compared to the other dietary and gender conditions. Using smaller particles to remove hepatic TG would be more demanding on PC levels, since more lipoprotein surface area would be required for the secretion of smaller quantities of core neutral lipids. Furthermore, it is clear from our studies that male HF/HC fed animals had the highest demands for total plasma PC as compared to the other study groups (Fig. 3.8A).

Interestingly, radiolabeled LPC and SM were specifically targeted for VLDL secretion in female chow fed animals (Fig. 3.7). Specific activity for these phospholipids in the plasma decreased in response to both the HF/HC diet, as well as the male gender (Fig. 8C). Again, specific activities were not necessarily significant within the groups but the trends were such that the difference between female chow and male HF/HC were significant.

Our observations in the liver, bile and plasma suggest that PEMT derived phospholipids are more readily made in females as compared to males, and most favorably in the chow fed conditions. Unfortunately, although Triton WR 3119 inhibits lipoprotein lipase it is not known to inhibit the uptake of apoE containing lipoproteins via the LDLR. Furthermore, the contribution of hepatic PC to the HDL fraction is unknown, and Triton WR 3119 does not inhibit uptake of these particles. Since mice are generally considered HDL animals (44), this constitutes a very significant pool of PC. Finally, there is the additional limitation of not being able to distinguish newly synthesized, and pre-existing PC in any of the tissues or fluids analyzed. One way to address this problem would be to perform a double labeling experiment with both Met and choline to study the targeting of all newly synthesized PC. This too has its limitations however, since the Kennedy pathway produces PC in all tissues that could be incorporated into HDL particles. Since it has not yet been established how much HDL derived PC is taken up by the liver this could interfere with the analysis.

In conclusion, this story remains incomplete and further studies will be needed. However, techniques in the whole animal do not currently exist to address these questions. While the studies shed some light on PC metabolism in males/females in response to different diets, they still do not explain why the *Pemt*^{-/-} male mice were unable to tolerate the HF/HC diet (Chapter 2). Perhaps with the combination of the male gender and the *Pemt*^{-/-} genotype, CT activity was already saturated in the active fraction under chow conditions and little protein was available for further activation under the challenge of the HF/HC diet.

Our data shows preliminary evidence that the two predominant pathways for PC synthesis in the liver do respond differently to both gender and diet. Yet, like most research, it has raised more questions that need to be investigated. Do female mice have

different PC requirements compared to males (pregnancy and lactation)? What are the mechanisms for the differences observed for CT activation and which of the 3 isoforms are involved? Is PEMT derived PC produced by the ER targeted to a different pool than the PC made in the MAM? Both gender and diet seem to play an important role in regards to the development of cardiovascular disease. Understanding the mechanisms of PC production for VLDL synthesis will help to elucidate the roles these two risk factors play in cardiovascular disease.

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Chapter 4

**Secretion of ApoB100 Containing Lipoproteins is
Impaired from Hepatocytes Isolated from *Pemt*^{-/-} Mice**

4.1 Introduction

In our *in vivo* studies outlined in Chapter 2, it was clear that PEMT derived PC was necessary for the secretion of apoB100 containing VLDL from male mice fed a HF/HC diet. However, trying to determine the exact mechanism for the effects observed are difficult to perform in the whole animal due to the many potential variables that need to be taken into account (Chapter 3). In the present study, hepatocytes isolated from *Pemt*^{-/-} and ^{+/+} (1) mice are isolated and used as a model system to study the role of PEMT activity for the secretion of VLDL.

Hepatocytes are a common model system for examining rates of VLDL secretion and have been used in the past to study the role of PEMT derived PC (2). Data obtained from rat hepatocytes treated in choline, and Met deficient conditions provided the first clear evidence that PC is essential for VLDL secretion (3, 4). Treatment of rat hepatocytes with DZA, an inhibitor of all methylation reactions, resulted in no change in VLDL secretion suggesting that PEMT derived PC is not required (5). Later, it was demonstrated that even though PEMT activity was inhibited by >95%, some PEMT derived PC was still targeted for VLDL secretion (6). Other studies using the PEMT inhibitor bezafibrate, showed that secretion of apoB48 containing lipoproteins shifted from the VLDL fraction to the LDL and HDL fractions (apoB100 secretion remained normal) (7). The last study conflicts with the results observed *in vivo* in the *Pemt*^{-/-} mouse where apoB100 secretion was decreased (Chapter 2). DZA inhibits all methyltransferase reactions that use AdoMet as a substrate (8) and bezafibrate is a hypolipidemic agent and a well known peroxisomal proliferator (9). Therefore, these two compounds affect the activities of many enzymes so it was always questionable if the effects were specific for PEMT activity.

Using hepatocytes from the *Pemt*^{-/-} mouse is an ideal model system since only PEMT activity is affected and so there are no other variables. In this study, the specific loss of PEMT activity results in a reduction of apoB100 secretion. This reduction in VLDL secretion also is observed as a decrease in TG secretion. The advantage of the hepatocyte system is that in future studies, it is relatively easy to look at intracellular apoB100 and at what stage VLDL production halted.

4.2 Materials and Methods

4.2.1 Materials: The Hank's Balanced Salt Solutions used during the liver perfusions was obtained from Gibco BRL, Life Technologies, Grand Island, NY. The hepatocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) also from Gibco BRL, Life Technologies. The collagen, collagenase, insulin, BSA, oleate, KBr and Protein A Sepharose CL-4B were all purchased from Sigma. Silica gel G60 plates for TLC were from Merck, while the PC and TG (triolein) standards were from Avanti Polar Lipids. Both [^3H]-oleate and [^{35}S]-Met radiolabels were obtained from Amersham Pharmacia. The sheep anti-human apoB antibody used in immunoprecipitations was purchased from Boehringer Mannheim. Finally, all other chemicals and reagents were purchased from standard commercial sources.

4.2.2 Isolation of Hepatocytes: For all experiments, 12-20 week old male *Pemt*^{-/-} and *Pemt*^{+/+} mice that had been maintained on a regular rodent chow (PICO Lab Rodent Diet 20) diet were utilized (Chapter 2). Primary hepatocytes were isolated from the livers using the collagenase perfusion technique as previously described (10,11). The cells were always isolated and plated in DMEM containing 17% FBS and 0.01 mg/ml insulin. During isolation, the cells were pelleted and washed 3x and passed through both coarse and fine filters to remove any debris. The primary cultures were plated at a density of 2×10^6 cells on collagen coated 60 mm plates. The cells were allowed to adhere for 2 hr, after which the media was replaced to remove all the dead cells. In all cases, the hepatocytes were isolated in the late afternoon and allowed to settle overnight in the above culture media. For all the experiments, the cells were washed 2x over a minimum 1 hr period in serum and hormone free DMEM followed by the experimental procedure.

4.2.3 PEMT Assays: Hepatocytes isolated from *Pemt*^{+/+} mice were monitored for loss of PEMT activity. Following the 1 hr rinse with serum free DMEM, the hepatocytes were cultured in 2 ml serum free DMEM. At time points 0, 6, 12 and 18 hr the cells were harvested. The cells were scraped into PBS and sonicated for 20 sec each. Protein

determination was performed using the Pierce BCA kit using BSA as a protein standard. PEMT assays were performed on the samples as previously described using PMME as a substrate (12).

4.2.4 Secretion of Total Lipid Mass: For these experiments, many cells were required and so the hepatocytes isolated from 2 animals for each genotype were pooled and plated together. Prior to the experiment, the cells were rinsed 2x over a 1 hr period with serum free DMEM. Each plate was then incubated in 2 ml serum and hormone free DMEM either in the presence of 0.75 mM oleate, 1% BSA or 1% BSA alone. The cells and media were harvested at 0, 6, and 12 hr. For each sample, 4 plates were pooled.

Lipids were extracted from the media using a modified Bligh-Dyer protocol (13). Following the extraction the entire sample was loaded onto TLC and resolved using the double solvent system described in Chapter 2 in order to separate both the phospholipids and the neutral lipids. The bands were visualized with iodine vapour and both TG and PC bands were scraped. PC mass was determined via the lipid phosphorus Malachite Green assay (14) and TG mass was measured using the hydroxylamine procedure (15).

The cells were scraped and pooled into 8 ml PBS and spun to remove any dead cells. The cellular pellet was then resuspended in 4 ml PBS and sonicated. Protein assays were performed and lipids were extracted from 1 mg of protein using the Bligh-Dyer protocol (13). The lipids were resolved by TLC, as described above, and PC and TG mass were determined.

4.2.5 Radiolabeling and Density Fractionation of Secreted Lipids:

Hepatocytes from one male mouse for each genotype were isolated, plated and prepared as described previously. After an overnight incubation the cells were rinsed 2x over a 2 hr period with serum free DMEM. Cells were then radiolabeled with 10 μ Ci of [^{3} H]-oleate (Amersham) in the presence of 0.75 mM oleate, 1% BSA for 12 hr. Media and cells were harvested, and lipoproteins were isolated by ultracentrifugation (16). Briefly, 1.4 ml media was mixed with 0.7g KBr and then loaded at the bottom of a Quick-seal tube and overlaid with 0.9% NaCl (17). The samples were centrifuged at 416 000 g for 1 hr in a Vti 65.2 Beckman rotor (17). After, 0.5 ml samples were collected

from the bottom and each fraction underwent a lipid extraction (13) as well as TLC using the two solvent system. Bands corresponding to TG and PC were scraped and measured by counting in the presence of scintillant.

4.2.6 Steady State Secretion of ApoB from *Pemt*^{-/-} and ^{+/+} Hepatocytes:

The hepatocytes from one male mouse for each genotype were isolated, plated and prepared as described above. After an overnight incubation the cells were rinsed 2x over a 2 hr period with serum and Met free DMEM. Cells were then radiolabeled with 200 μ Ci [³⁵S]-Met for 4 hr, in the presence of either 0.75 mM oleate, 1% BSA or 1% BSA alone in Met free DMEM. Media and cells were harvested, and lipoproteins were isolated via ultracentrifugation as described above, except only 1.3 ml media was used, mixed with 100 μ l fresh mouse or rat plasma (isolation performed in the presence of a total concentration of 1 μ M benzamidine). ApoB48 and B100 were isolated via immunoprecipitation using an anti-human apoB antibody. To each 500 μ l density fraction, 55 μ l of 10x immunoprecipitation buffer [1.5 M NaCl, 0.5 M Tris pH 7.4, 50 mM EDTA, 5% Triton X-100 (v/v) and 15 SDS (w/v)] (17) and 5 μ l anti-human apoB was added and the samples were mixed overnight at 4°C. Each sample was mixed with 50 μ l Protein A Sepharose CL-4B in PBS (1:1) for 1 hr. The Sepharose was pelleted for 5 min at maximum speed in a microfuge and washed 2x with 1x immunoprecipitation buffer. The pellets were boiled in 1x sample buffer [125 mM Tris/HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), 10% β -mercaptoethanol (v/v), and 0.02% Bromophenol Blue] (18) and resolved on a 5% SDS-PAGE gel. The gels were stained with Coomassie stain, soaked in Amplify (Amersham, Pharmacia), dried and exposed to film. Quantitative analysis of the bands was determined using Image Gauge v3.0 software by Fuji and units were divided by cellular protein.

4.3 Results:

4.3.1 PEMT Activity Decreases with Time: Previous studies on rat hepatocytes indicated that PEMT activity is not stable in primary hepatocytes (D.E. Vance, unpublished results). Therefore, in order to organize the following experiments properly, it was necessary to determine the stability of PEMT activity in mouse primary hepatocytes. Hepatocytes were isolated from *Pemt*^{+/+} mice, plated and allowed to settle overnight as described in section 4.2.2. In the morning, the cells were rinsed with serum free DMEM and then incubated over several time points. The cells were then collected and assayed for PEMT activity. As shown in Figure 4.1, PEMT activity was substantial at the 0 time point indicating that the overnight incubation was not detrimental to the enzyme.

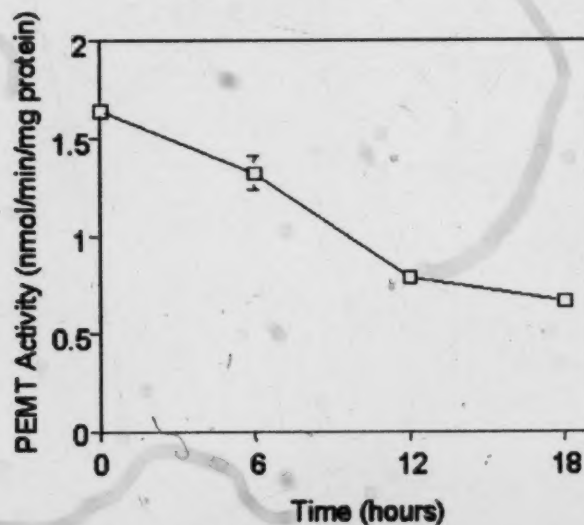


Figure 4.1: PEMT Activity is not Stable in Cultured Primary Mouse Hepatocytes

Hepatocytes were isolated from *Pemt*^{+/+} mice in the late afternoon and were allowed to settle overnight. The next morning, the plates were rinsed 2x over 1 hr with serum and hormone free DMEM. The hepatocytes were then cultured in DMEM alone for 0, 6, 12 and 18 hr. The cells were harvested, measured for protein, and then assayed for PEMT activity. Shown are the results of 2 separate preparations, with 1-3 plates for each time point for each preparation.

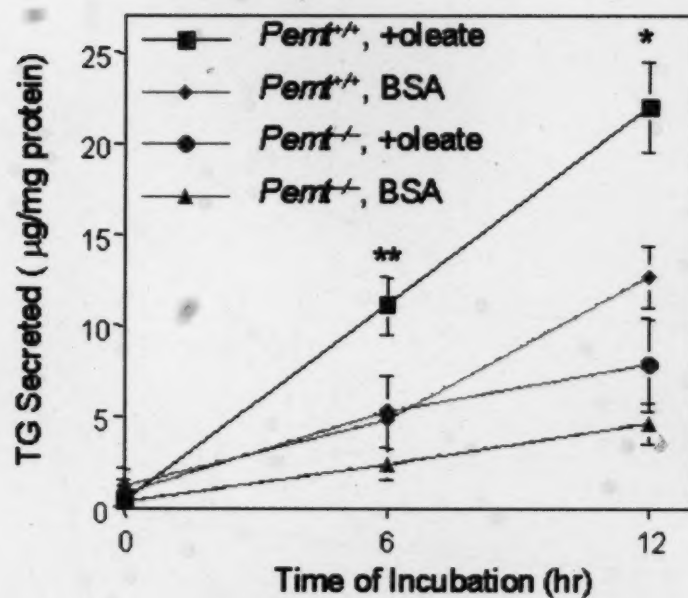
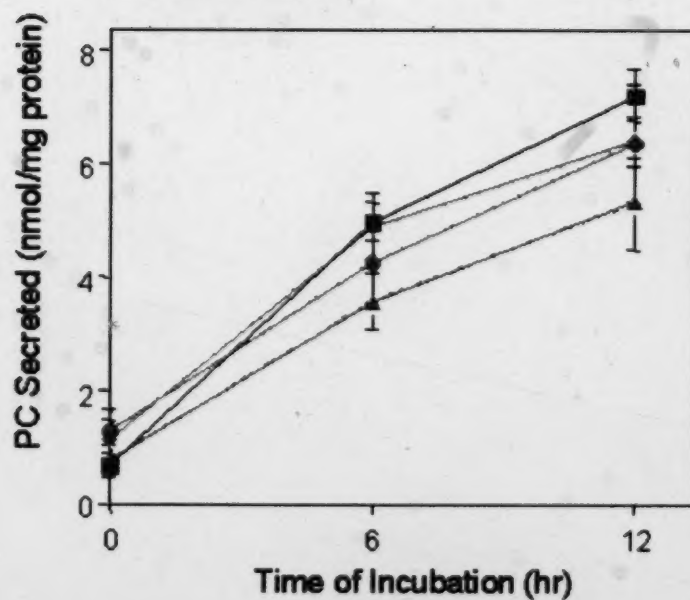
However, after 12 hr in serum free DMEM, PEMT activity was only half of that observed at the 0 time point. By 18 hr, PEMT activity had decreased even further. Therefore, from this experiment it was established that additional experiments should not extend beyond a 12 hr time point.

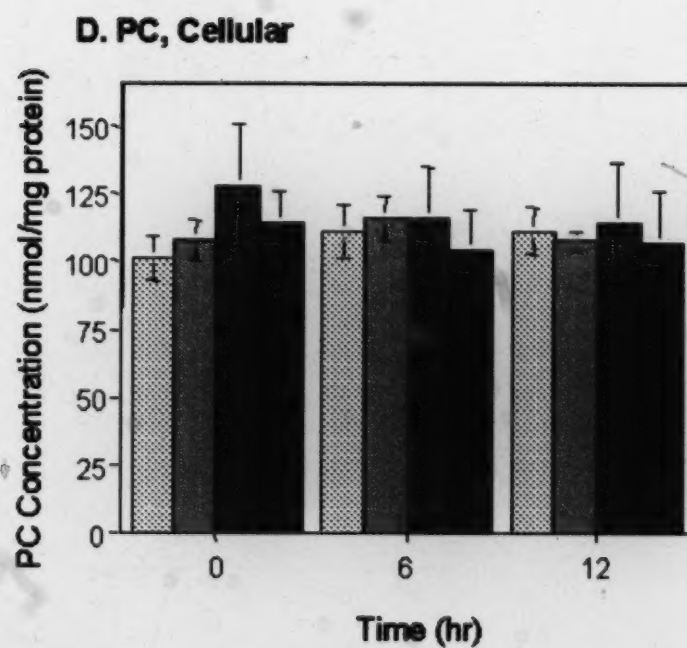
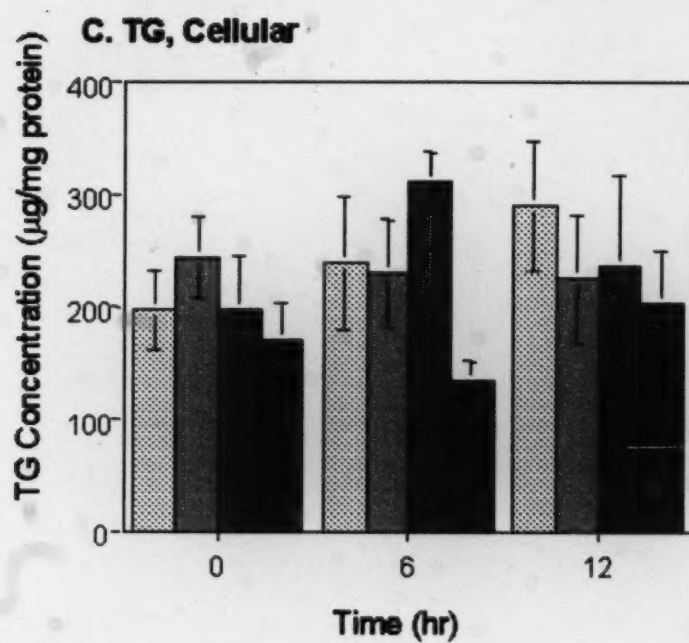
4.3.2 TG Secretion is Impaired from *Pemt*^{-/-} Hepatocytes: Next, we were interested as to whether or not PEMT activity affected lipid secretion. Hepatocytes were isolated from both *Pemt*^{+/+} and ^{-/-} mice as described in section 4.2.4. After settling overnight and a 1 hr rinse in the morning, the hepatocytes were incubated either in the presence or absence of 0.75 mM oleate for 0, 6 and 12 hr. At each time point, the media and cells of 4 plates were combined for analysis. As shown in Figure 4.2A, the absence of PEMT activity resulted in an ~50% decrease in TG secretion from oleate treated cells, as compared to hepatocytes isolated from *Pemt*^{+/+} animals. Secretion of TG mass was lowest from cells isolated from *Pemt*^{-/-} mice treated with BSA alone, but it was not significantly lower than BSA treated cells from *Pemt*^{+/+} mice. Interestingly, the decrease in TG secretion observed for hepatocytes from *Pemt*^{-/-} mice did not translate into a decrease in total PC secretion (Fig. 4.2B).

Although TG secretion was low in the oleate treated *Pemt*^{-/-} hepatocytes, compared to their *Pemt*^{+/+} counterparts, there was no observable accumulation of TG mass in these cells at any time point (Fig. 4.2C). Why TG does not accumulate in these cells is puzzling. As well, no significant changes were observed in total cellular PC mass suggesting that the Kennedy pathway is sufficient to maintain normal PC levels in the cells isolated from mice (Fig. 4.2D).

Figure 4.2: TG Secretion is Impaired from *Pemt*^{-/-} Hepatocytes Treated with Oleate

The hepatocytes from two animals for each genotype were isolated, pooled and plated. The cells were allowed to settle overnight and in the morning were rinsed 2x over a 1 hr period with serum and hormone free DMEM. The plates were then incubated in 1% BSA alone, or 0.75 mM oleate, 1% BSA for the times indicated. For each sample, the media or cellular contents of 4 plates were combined. The lipids were extracted from the media using a modified Bligh-Dyer protocol, resolved by TLC and both TG (A) and PC (B) mass were determined. The cells were scraped and sonicated in PBS, protein was measured, lipids were extracted, resolved by TLC and TG (C) and PC (D) masses were determined.  *Pemt*^{+/+}, with oleate  *Pemt*^{+/+}, BSA alone  *Pemt*^{-/-}, with oleate  *Pemt*^{-/-}, BSA alone (applies to figures C and D only). The data shown represents the average of 3 separate experiments, +/- S.E.M. **P* < 0.005 and ***P* < 0.05 based on a Student's t-test.

A. TG, Secreted**B. PC, Secreted**



4.3.3 Secretion of VLDL is Impaired from Hepatocytes Isolated from *Pemt*^{-/-}

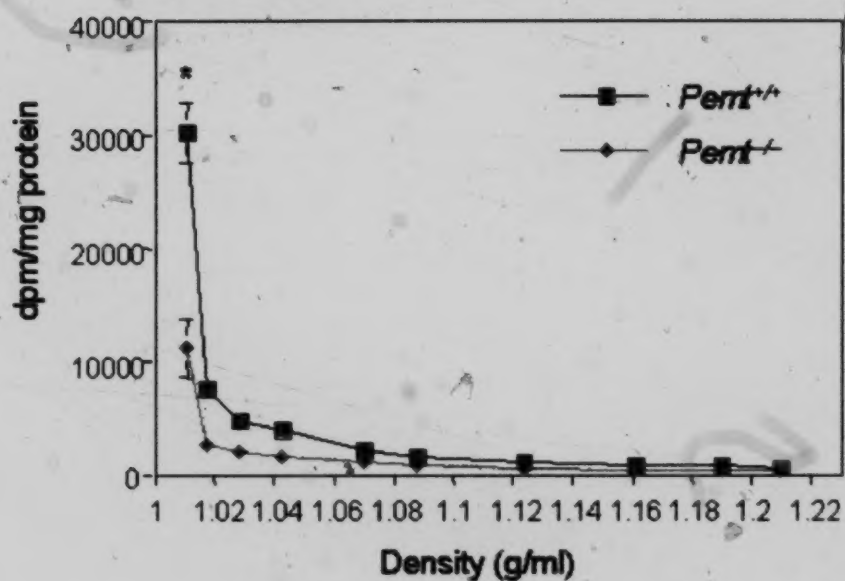
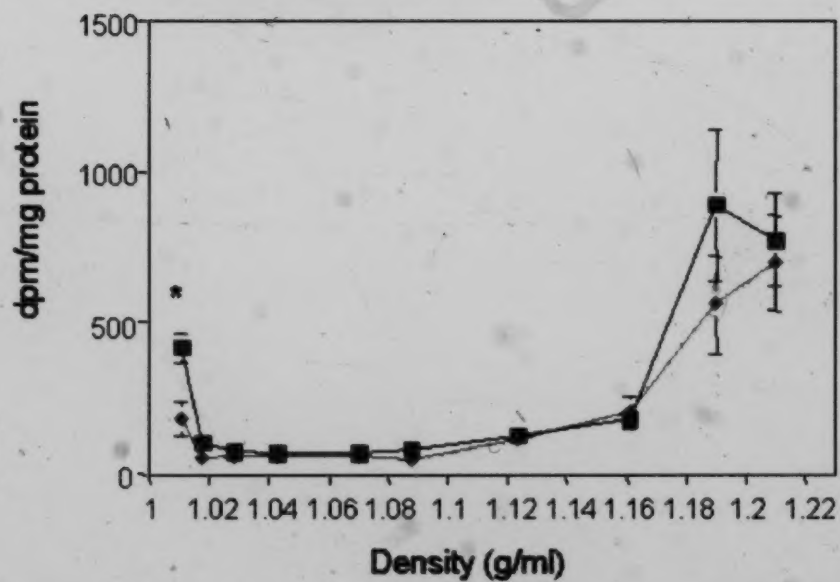
Mice: Experiments performed by Nishimaki-Mogami *et al.* (7) in rat hepatocytes demonstrated that inhibition of PEMT activity resulted in no effect on total apoB secretion. However, they did observe a shift in apoB48 containing lipoproteins from the VLDL fraction to the LDL/HDL fractions. Furthermore, *in vivo* lipoprotein profiling of radiolabeled PC (Chapter 3) showed that diet and gender has an affect on the size of lipoproteins secreted from the liver. In male, HF/HC fed *Pemt*^{+/+} mice, there was a shift in radiolabeled PEMT derived PC from the VLDL fraction to the HDL fraction. Using smaller particles to secrete TG is not efficient since the neutral core of these particles is small, while the total surface area would still be high requiring significant PC. Because of these previous results it was necessary for us to examine the lipoprotein profile of lipids secreted from the *Pemt*^{-/-} and ^{+/+} hepatocytes.

Hepatocytes isolated from *Pemt*^{-/-} and ^{+/+} males were incubated for 12 hr in serum free DMEM with 0.75 mM oleate, 1% BSA and 10μCi [³H]-oleate. The media was removed from the cells and was separated into lipoprotein fractions. Distribution of radiolabel was analyzed for both secreted TG and PC. In Fig. 4.3A it is shown that secreted VLDL TG was significantly decreased from the cells isolated from the *Pemt*^{-/-} animals compared to ^{+/+} mice. We did not observe a shift in the distribution of radiolabeled TG to smaller particles. Although there was no observed change in secreted PC mass (Fig. 4.2B), separation of radiolabeled PC into the lipoprotein fractions showed a significant decrease in the VLDL fraction of *Pemt*^{-/-} hepatocytes (Fig. 4.3B). The majority of radiolabeled PC was isolated in the HDL fractions where there were no significant differences observed.

4.3.4 ApoB100 Secretion is Impaired from *Pemt*^{-/-} Hepatocytes: In our *in vivo* diet studies we demonstrated that in male, *Pemt*^{-/-} mice fed a HF/HC diet, apoB100 secretion was specifically impaired (Chapter 2). Nevertheless, total fasted apoB48 and B100 levels were low in these animals suggesting increased catabolism of apoB48 containing particles. Here we were interested if apoB100 secretion was also specifically impaired from the hepatocytes isolated from *Pemt*^{-/-} animals. Each 60mm plate of isolated hepatocytes was incubated in serum and Met free DMEM for 4 hr in the presence

Figure 4.3: VLDL Secretion is Decreased from *Pemt*^{-/-} Hepatocytes

Hepatocytes from male *Pemt*^{-/-} and ^{+/+} mice were isolated, plated and allowed to settle overnight. In the morning the cells were rinsed 2x over a 2 hr period with serum and hormone free DMEM. The cells were then incubated for 12 hr in serum free DMEM containing 1% BSA, 0.75 mM oleate and 10 μ Ci [³H]-oleate. Secreted lipoproteins were separated via ultracentrifugation and each fraction underwent lipid extraction. The lipids were resolved by TLC, scraped and radioactivity was measured by liquid scintillation counting. Measurements were then normalized to total cellular protein. The data is shown as the average of 3 experiments, each performed in duplicate, $\pm$ S.E.M. **P* < 0.05 based on a Student's t-test.

A. Triglyceride**B. Phosphatidylcholine**

of 200 μCi [^{35}S] Met. Afterwards, the media was centrifugated to separate the lipoproteins and the apoB proteins were immunoprecipitated from each fraction. ApoB100 secretion from the hepatocytes was specifically decreased while apoB48 secretion was normal, as expected from our *in vivo* diet studies (Fig. 4.4). Quantitation of the results indicated that apoB100 secretion was only significantly impaired in *Pemt*^{-/-} hepatocytes treated with oleate (Fig. 4.5B,C). In the absence of oleate, loss of apoB100 secretion from the *Pemt*^{-/-} cells was inconsistent resulting in no significant difference. Quantitation of apoB48 bands indicated no differences in secretion or distribution (Fig. 4.5D,E).

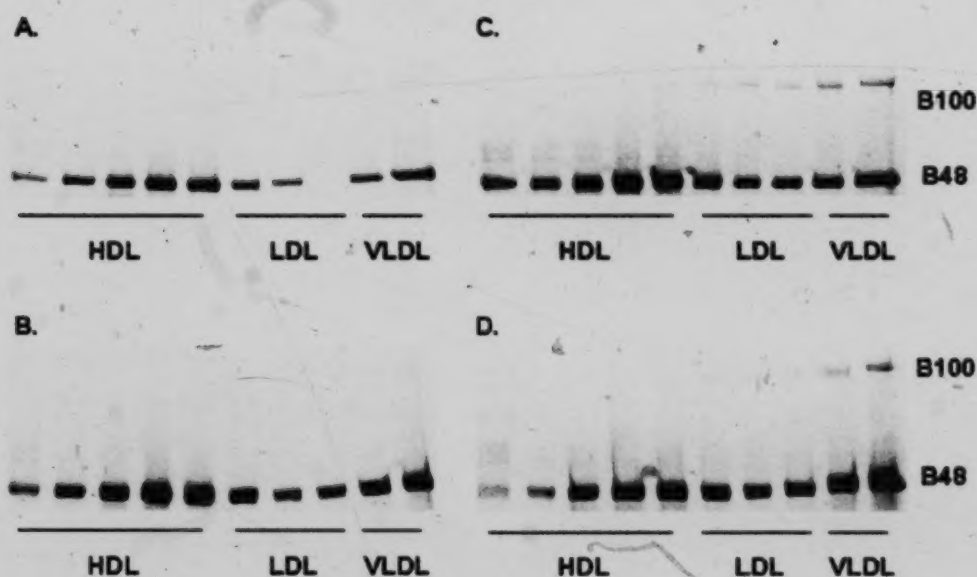
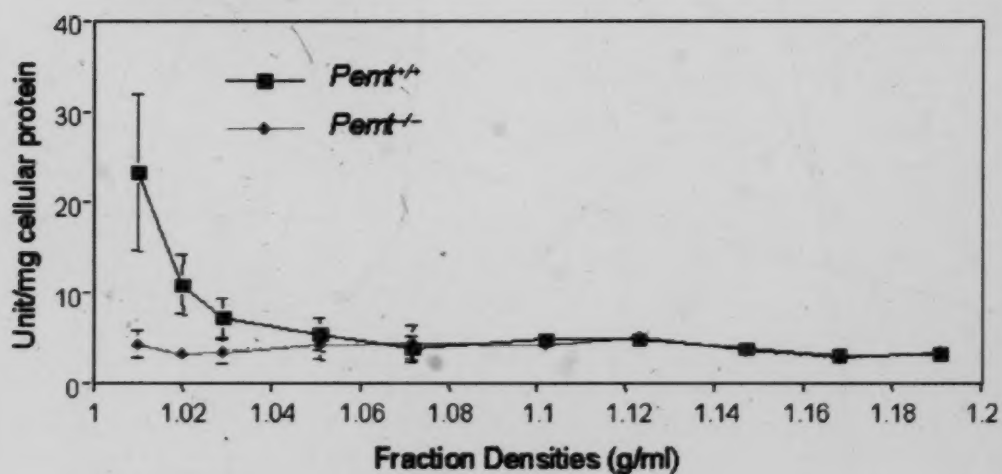
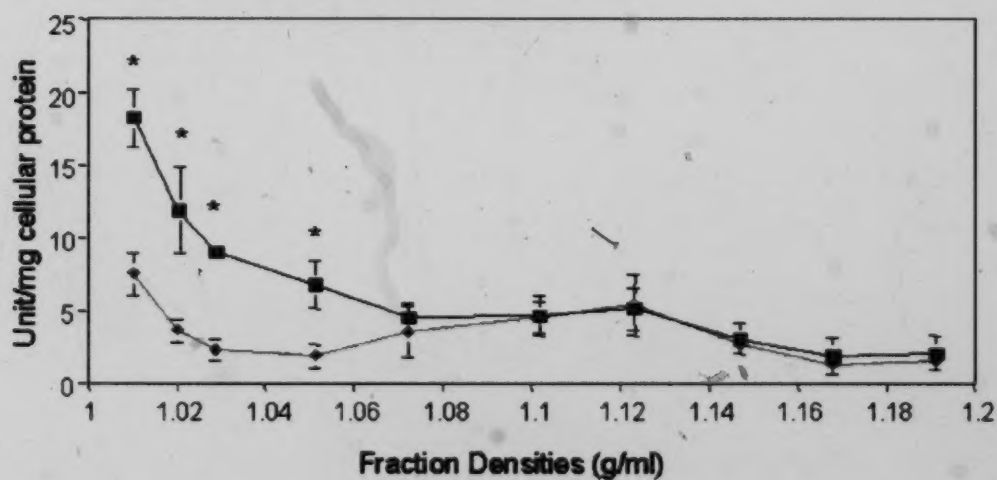


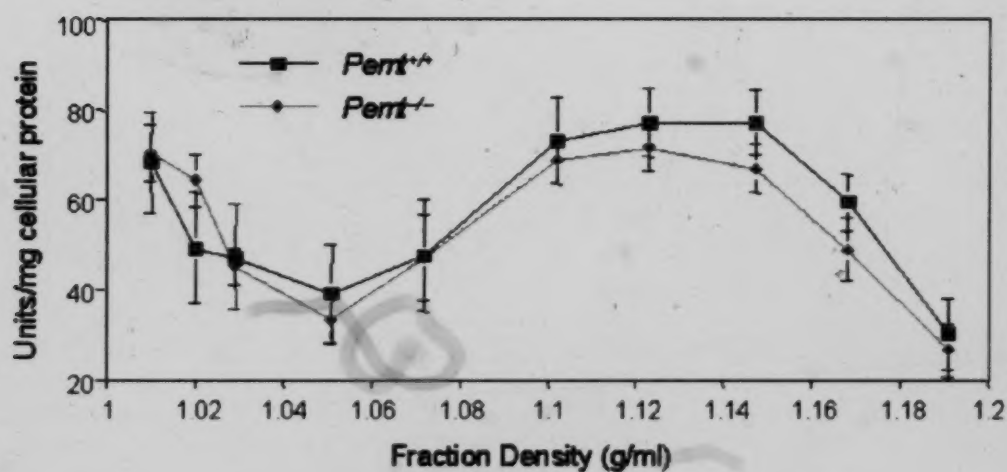
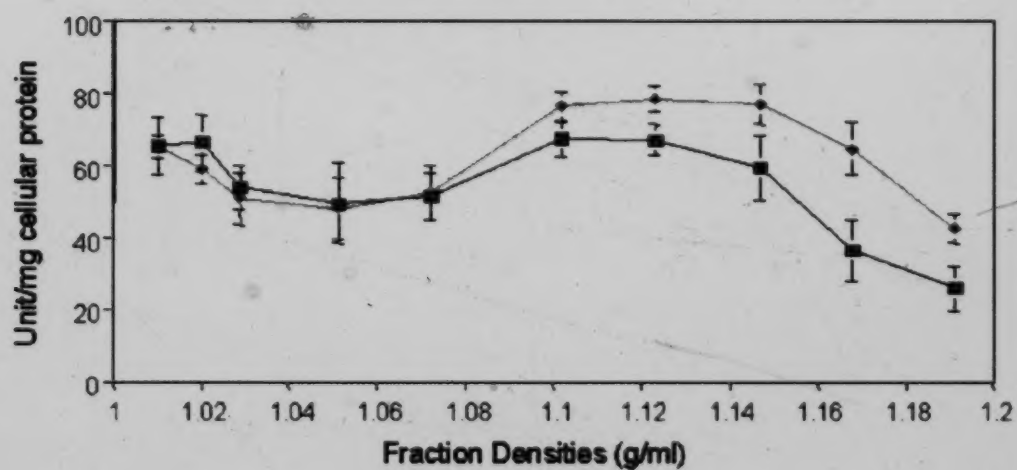
Figure 4.4: ApoB100 Secretion is Impaired from *Pemt*^{-/-} Hepatocytes

Shown is an example of secreted apoB100 and apoB48 proteins from hepatocytes isolated from *Pemt*^{-/-} (A,B) and *+/+* mice (C,D). Cells were treated with 200 μCi [^{35}S] Met for 4 hr in the presence (B,D) or absence (A,C) of 0.75 mM oleate.

Figure 4.5: ApoB100 Secretion is Impaired from Oleate Treated Hepatocytes, Isolated from *Pemt*^{-/-} Mice

Hepatocytes from *Pemt*^{-/-} and ^{+/+} mice were plated and allowed to settle overnight. The next morning, the cells were rinsed 2x over 1 hr with serum, hormone and Met free DMEM. The cells were then incubated for 4 hr in serum, hormone and Met. free DMEM containing either 1% BSA alone or with 0.75 mM oleate, and 200 μ Ci [³⁵S]-Met. The media from the plates was then separated into density gradient fractions and apoB100 and apoB48 proteins were immunoprecipitated from each fraction. The immunoprecipitates were resolved on a 5% SDS-PAGE gel. The gels were soaked in Amplify, dried and exposed to film. Quantitative analysis was performed using Image Gauge v3.0 software from Fuji and the units were normalized to total cellular protein. The data shown represents the average of 4 separate experiments, with duplicates, $\pm$ S.E.M. **P* < 0.05 based on a Student's t-test.

A. ApoB100, 1% BSA**B. ApoB100, 0.75 mM oleate, 1% BSA**

C. ApoB48, 1% BSA**D. ApoB48, 0.75 mM oleate, 1% BSA**

4.4 Discussion

We examined the role of PEMT derived PC in VLDL secretion using the primary hepatocyte model. The primary hepatocyte model has certain advantages for studying lipid and lipoprotein metabolism. They maintain many of the liver's functions in an isolated system without interfering variables such as plasma lipids. Furthermore, in using cultured primary hepatocytes, one can investigate the response of one liver to a number of conditions in a controlled setting (2). Unfortunately, the disadvantage of using primary hepatocytes is that over time they lose many enzymatic activities and they have a limited life span. Therefore, it is always important to examine the activity, and/or expression of the protein of interest. In the case of PEMT, allowing the cells to settle overnight did not result in a loss of activity (Fig. 4.1). However, after a 12 hr incubation in serum free DMEM, 50% of the PEMT activity was lost and it was concluded that further experiments should not extend beyond a 12 hr time point. It is interesting to note that PEMT activity was lost only with the removal of serum from the media. This suggests that PEMT expression is potentially regulated by a serum-derived factor and this observation should perhaps be considered in future experiments.

When hepatocytes isolated from *Pemt*^{-/-} and ^{+/+} cells were treated only with 1% BSA, no significant difference was observed in TG mass secretion (Fig. 4.2A). Yet, in attempting to stimulate further VLDL secretion by treating the cells with 0.75 mM oleate, the cells isolated from the *Pemt*^{-/-} mice were only capable of secreting ~50% of the TG mass secreted by the *Pemt*^{+/+} hepatocytes. These results concur with the diet studies performed in the whole animal. Plasma TG levels and VLDL secretion were only impaired in the male *Pemt*^{-/-} mice fed a HF/HC diet, while they were normal under chow dietary conditions (Chapter 2). The observation that TG secretion was not significantly impaired from the *Pemt*^{-/-} hepatocytes when treated with BSA alone suggests that PEMT activity is required for normal TG secretion only under stressful conditions. PC secretion however was normal for the hepatocytes isolated from the *Pemt*^{-/-} mice compared to the ^{+/+} animals in the absence or presence of oleate (Fig. 4.2B).

Next, we examined the lipoprotein profiles of secreted TG and PC from oleate treated *Pemt*^{-/-} and ^{+/+} mice. The decrease in secreted TG was specifically accounted for

by a loss in the VLDL fraction (Fig. 4.3A). Interestingly, although there was no observed difference in secreted PC mass between the two genotypes, secreted VLDL PC was significantly diminished from the cells isolated from *Pemt*^{-/-} mice (Fig. 4.3B). Still, the majority of PC secreted by the mouse hepatocytes was found in HDL sized particles. Therefore, the difference observed in the VLDL fraction might not have been adequate to cause a significant difference in total PC mass. The results obtained from this experiment conflict with the results obtained by Nishimaki-Mogami *et al.* (7) where a shift to smaller particles was observed with the onset of PEMT inhibition. Still the inhibitor used in those studies, bezafibrate, is not a PEMT specific inhibitor and can potentially affect many lipid-regulating enzymes, which could have potentially affected their results (9). Furthermore, the bezafibrate studies were not performed with oleate, to stimulate VLDL secretion, which could also account for any conflicting results with this study.

In the *in vivo* diet studies, only apoB100 secretion was impaired in the male *Pemt*^{-/-} animals, while apoB48 secretion was normal. Using the hepatocyte model, the same observation was made, only apoB100 secretion was impaired from the cells isolated from the *Pemt*^{-/-} mice (Fig. 4.4, 4.5). The decrease in apoB100 secretion was consistent only in the *Pemt*^{-/-} hepatocytes treated with oleate, again confirming the role PEMT has in VLDL secretion exclusively during a high fat challenge.

Although this study demonstrates that PEMT derived PC does play a role in VLDL secretion from hepatocytes, it does not delve into what that role is. The fact that PEMT only appears to be necessary for VLDL secretion during a high fat challenge suggests that perhaps during such conditions, there is simply not enough PC for VLDL secretion (after adequately supplying the needs of the cell). In our *in vivo* diet studies, the female *Pemt*^{-/-} animals maintained normal VLDL secretion when fed either a chow or HF/HC diet (Chapter 2). However, a 30-40% decrease in plasma cholesterol and PC was observed for both dietary conditions and this decrease was located in the HDL fraction. One hypothesis that arose from this observation was that the livers of the female *Pemt*^{-/-} mice were compensating for the loss of PEMT by either taking up more, or secreting less HDL lipid. In all of our hepatocyte experiments, the cells were incubated in serum free media excluding a potentially important source of PC. Preliminary studies treating *Pemt*

^{-/-} hepatocytes with LPC, in order to rescue TG secretion in the presence of oleate, have been so far inconclusive.

Another possible role PEMT could be playing during VLDL secretion is mobilization of lipid stores. It is well established that the majority of cellular TG is localized in cytosolic stores that are inaccessible for VLDL secretion (19). To be accessible for VLDL secretion, the TG must reach ER luminal stores. Two enzymes, TGH and AADA, are presently hypothesized to be responsible for hydrolyzing cytosolic TG stores to DAG/MAG, which cross the ER bilayer and are reacylated by diacylglycerolacyltransferase to TG (19). Nevertheless, another known contributor to luminal TG stores is hydrolysis of bilayer PC (20). Interestingly, along with TGH, PEMT has also been found in rat liver fat droplets (21). Perhaps, PEMT produces PC specifically for hydrolysis and conversion to TG, or maybe it provides membrane for enclosing TG lipid stores.

With regards to this hypothesis, another mouse model where a specific decrease in only apoB100 secretion was observed is the conditional, liver specific MTP knockout (22). Interestingly, in addition to decreased apoB100 secretion, the loss of MTP expression resulted in a significant decrease in luminal TG droplets in the ER and the Golgi (22). A number of studies have suggested that MTP plays a role not in the mobilization of TG, but the transfer of newly reacylated TG to the VLDL accessible pool (23-25). The similarity between the MTP conditional knockout and the *Pemt*^{-/-} mouse with regards to apoB100 secretion, may provide clues to the role PEMT derived PC plays in VLDL secretion. As described above, perhaps PEMT is playing a role in the maintenance of the ER luminal, VLDL accessible TG pool when the hepatocyte is presented with a large influx of fatty acids. It is well established that hepatic secretion of apoB100 is far more dependent on TG availability than secretion of apoB48 (26, 27). Therefore the synthesis and secretion of these particles is far more sensitive to potential fluxes in ER luminal TG levels.

It is important to note that although the *Pemt*^{-/-} hepatocytes treated with oleate were secreting only 50% TG mass compared to cells from ^{+/+} animals, they did not accumulate intracellular TG as expected (Fig. 4.2C). The reason for this discrepancy is not at all understood. It would be worthwhile in future experimentation to follow the fate

of oleate in these cells by measuring DAG, free fatty acid levels as well as phospholipid turnover. Equivalent levels of intracellular radiolabeled TG and PC in both *Pemt*^{-/-} and ^{+/+} hepatocytes was determined when the cells were treated with [³H]-oleate (data not shown). This result suggests that uptake of oleate was not impaired in the hepatocytes isolated from the *Pemt*^{-/-} mice.

Previous choline deficiency studies in rats demonstrated that the Kennedy pathway is required for normal VLDL secretion. Subcellular fractionation and electron microscopy studies demonstrated that in a CD state VLDL particles are produced but degraded in a post-ER site (28). Studies accomplished *in vivo* (Chapter 2) and here confirm that PEMT derived PC is required for secretion of apoB100 containing particles under the challenge of high fat. Therefore, the next stage is to determine at what point apoB100 particle formation is discontinued and determine the reason why.

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Chapter 5

Phosphatidylethanolamine *N*-methyltransferase Activity Contributes Significantly to Plasma Homocysteine Levels in Mice

*This work was a collaborative effort with Dr. J. Brosnan and Ms. L.M. Stead
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5.1 Introduction

Mild hyperhomocysteinemia has been demonstrated to be an independent risk factor for cardiovascular disease (1-3). Hcy is a toxic, sulfur-containing, nonproteinogenic amino acid produced during Met metabolism (4, 5). AdoMet is an essential methyl donor for almost all methyltransferase reactions after which it becomes an AdoHcy molecule (Fig. 1.5). When AdoHcy accumulates in excess it is hydrolyzed to Hcy by the action of AdoHcy hydrolase (4, 5). Hcy has three possible metabolic fates. It can be remethylated to Met using either *N*-5 methyltetrahydrofolate, or betaine, as the methyl donor. When there is excess AdoMet, Hcy enters the transsulfuration pathway to eventually produce Cys. Finally, Hcy can be released into extracellular fluids such as plasma and urine. Hyperhomocysteinemia is a result of an imbalance between Hcy production, and metabolism through either remethylation or transsulfuration (4, 5).

AdoHcy hydrolase activity favors the production of AdoHcy (4, 5). Therefore, in the presence of adenosine, an Hcy molecule will be rapidly converted to AdoHcy. AdoHcy is a potent inhibitor of all methyltransferase reactions that use AdoMet as a substrate (4, 5). Therefore part of the toxic effect of elevated Hcy is hypomethylation due to the accumulation of AdoHcy, particularly DNA hypomethylation, which can interfere with normal cell growth (4,5). Studies in the CBS^{+/−} mouse (CBS is the first step of the transsulfuration pathway) under a methyl-deficient diet suggested that a decrease in tissue AdoMet:AdoHcy ratios, due to elevated Hcy levels, resulted in global hypomethylation (6).

Endothelial cells are especially susceptible to the toxic effects of hyperhomocysteinemia. Experimental hyperhomocysteinemia in baboons over a 3 month period resulted in patches of vascular de-endothelialization stimulating excessive platelet recruitment (7). As well, studies in humans (8), CBS^{+/−} mice (9) and cell culture (10, 11) all concur that elevated Hcy levels result in endothelial dysfunction. Injury to the endothelial cell layer of the vascular wall contributes to the development of the atherosclerotic lesion since the intima is now readily accessible by lipoproteins and inflammatory cells (12). Furthermore, endothelial secretion of NO is an important vasodilator response, which is decreased with the loss of the vascular endothelial

monolayer (13). Studies in humans have specifically demonstrated that mild hyperhomocysteinemia impairs endothelial vasodilation (14,15). Furthermore, Hcy also potentially inhibits the expression of glutathione peroxidase, an anti-oxidant enzyme which sequesters oxidative species that otherwise also inactivate NO (16).

In contrast to the damaging effects to the vascular endothelial monolayer, elevated Hcy levels stimulate vascular smooth muscle cell growth, migration and their secretion of collagen (17, 18). Hcy also contributes to the recruitment of monocytes to the atherosclerotic lesion by stimulating the expression of monocyte chemoattractant protein-1 and interleukin-8 in both vascular endothelial cells and smooth muscle cells (19, 20). As well, Hcy treatment of macrophages in cell culture also results in enhanced expression of monocyte chemoattractant protein-1 (21) while treatment of monocytes with Hcy stimulates the expression of monocyte chemoattractant protein-1 receptor (22). Hcy has also been demonstrated to enhance binding of Lp(a) to fibrin (23). Finally, high intracellular Hcy concentrations enhanced expression of SREBP-1 in HepG2 cells resulting in an increase in the expression of a number of enzymes involved in the synthesis of cholesterol (24). These results were later repeated *in vivo* in the CBS^{+/+} mouse fed a diet to induce hyperhomocysteinemia (24).

Although Hcy presents all these effects that enhance the development of the atherosclerotic lesion it has not yet been elucidated if Hcy is a direct cardiovascular disease risk factor. Studies in patients with mild hyperhomocysteinemia, but without any other cardiovascular disease risk factors, were shown to be at no greater risk for disease compared to healthy individuals (25). In this context, it has been hypothesized that elevated plasma Hcy levels promote cardiovascular disease under conditions that already predispose an individual to vascular blockage (2, 26).

Interestingly, plasma Hcy levels can be easily and cheaply maintained by diet. Remethylation of Hcy to Met requires folic acid and vitamin B₁₂ while the transsulfuration pathway is dependent on sufficient dietary intake of vitamin B₆ (4, 5). Increasing dietary intake of folic acid is known to be the most effective means to decrease plasma Hcy concentrations (27, 28). Still, a number of studies have also demonstrated that an unhealthy lifestyle including smoking, excessive drinking, obesity, type II diabetes and an unhealthy diet all contribute to mild hyperhomocysteinemia (29-

36). More specifically, a study performed in rats suggests that hyperinsulinemia and hyperglycemia correlate with increased plasma Hcy levels (37). Experiments measuring the expression of methyltetrahydrofolate reductase and CBS in response to elevated glucose and insulin in rat liver, and HepG2 cells, have so far demonstrated conflicting results (37, 38). In contrast, rats made hyperglucagonemic displayed a 30% decrease in plasma Hcy specifically as a result of elevated expression of CBS, glycine *N*-methyltransferase and cystathionine γ -lyase (the second step of the transsulfuration pathway) (39). Taken together, these studies all demonstrate that elevated glucose intake and hyperinsulinemia are both detrimental for plasma Hcy levels.

It is well established that the enzymes of both the remethylation pathways and the transsulfuration pathway are important in regulating Hcy concentrations (4, 5). Genetic defects in these enzymes can lead to homocystinuria where Hcy levels are grossly elevated resulting in neurological, vascular and skeletal abnormalities (4, 5, 40). In addition, glycine *N*-methyltransferase is an important modulator of AdoMet:AdoHcy levels (4). Still, the source of mildly elevated plasma Hcy levels in people who are at high risk for cardiovascular disease has not been fully determined.

PEMT produces 3 AdoHcy molecules with every PC molecule it synthesizes (41). As well, our studies demonstrating that PEMT is involved with VLDL secretion in response to a high fat challenge (Chapter 2) made us question whether or not PEMT could be an important modulator of plasma Hcy levels as a function of diet. Hyperinsulinemia is known to elevate both Hcy (37) and VLDL secretion (42), suggesting a link between these two risk factors. In this study, we utilize the *Pemt*^{-/-} mouse to determine if PEMT contributes significantly to plasma Hcy and whether or not that can be manipulated as a function of gender or diet.

5.2 Materials and Methods

5.2.1 Materials: The semi-purified diet lacking a fat source utilized in the HF/HC diet was purchased from ICN. The Hank's Balanced Salt Solutions, DMEM and FBS used in isolating and culturing hepatocytes were from Gibco BRL, Life Technologies. The collagen, collagenase, insulin, BSA, oleate and guanidinoacetate utilized in the

hepatocyte experiments were all purchased from Sigma. For all the analyses performed by Dr. J. Brosnan's lab, all chemicals and supplies were obtained from Sigma unless otherwise noted.

5.2.2 In vivo Diet Experiments: The *Pemt*^{-/-} mouse colony had a mixed genetic background of 129/J and C57BL/6 and was maintained by homozygous breeding in a reversed 12-hr light/dark cycle (43). As described in Chapter 2, at the age of 12-14 weeks *Pemt*^{-/-} and ^{+/+} animals were fed *ad libitum* either a rodent chow, or a HF/HC diet for 3 weeks. The HF/HC diet was made as described in Chapter 2 (44). During the diet, the animals were held in regular cages containing bedding in a non-reversed 12 hr light/dark cycle. Following 3 weeks, the animals were fasted overnight and sacrificed in the early morning. Blood was collected in trace quantities of 250 mM EDTA, and spun in a microfuge to separate the plasma. The plasma was then frozen immediately in a -70°C freezer. The livers were also removed, briefly rinsed in ice cold PBS and then quick-frozen in liquid N₂. Plasma and liver samples were sent on dry ice to Dr. John Brosnan's lab at Memorial University, Newfoundland, for analysis.

5.2.3. Isolation and Treatment of Primary Hepatocyte Cultures: For all experiments, 12-20 week old male *Pemt*^{-/-} and ^{+/+} mice maintained on a regular rodent chow diet were utilized. Primary hepatocytes were isolated from the livers using a collagenase perfusion technique and were plated as described in Chapter 4 (45, 46). Again, 2 hr after plating, the cells were rinsed in order to remove any dead cells. Two different types of experiments were performed with the hepatocytes.

In the first experiment, cells were isolated and plated in the late afternoon and allowed to settle for 5 hr. The plates were rinsed 2x with serum free DMEM over a 1 hr period. The plates were then incubated for 18 hr in 1.5 ml serum free DMEM containing either 1% BSA alone or 0.75 mM oleate, 1% BSA. One set of triplicate plates was also incubated with 1.5 ml DMEM with 17% FBS. These stringent conditions (time and media volume) were chosen to optimize Hcy levels in the media. Afterwards, the media was collected and any dead cells were pelleted at maximum speed for 5 min in a microfuge (4°C). The media was then frozen at -70°C. The cells were scraped into 2 ml

PBS, and pelleted in a microfuge. The cells were frozen at -70°C and were later resuspended in PBS and protein was determined using the Pierce BCA kit using BSA as a protein standard.

In the second pilot experiment, the hepatocytes were isolated and plated in the morning. The cells settled over a 7.5 hr period after which they were rinsed 2x in serum free DMEM over a 1 hr period. The primary cultures were then incubated in 1.5, 2 or 3 ml serum free DMEM for 3, 6 and 12 hr time points. An additional set of plates was incubated in 3 ml serum free DMEM containing 200 μM guanidinoacetate. At each time point the media and cells were collected and processed as described above. All samples underwent analysis at the Brosnan lab at Memorial University.

5.2.4 Methionine and Cystathionine Measurements: Met and cystathionine concentrations were measured using reversed phase HPLC. The equipment used for these measurements were a Waters automated HPLC system including a Model 600E System Controller, a Model 712 WISP sample injector, a Model 420 Fluorescence Detector and a 743 Data Module. Briefly, following derivatization with O-phthalaldehyde the samples were injected into a Hypersil ODS-C18 column (4.6 x 150 mm). Amino acids were resolved over a 47 min linear gradient from Buffer A (100%) to Buffer B (100%) at a flow rate of 1.5 ml/min. Aminoethyl-cysteine was used as an internal standard. Buffer A contained a solution of 0.05 M sodium acetate and 0.05 M sodium phosphate (pH 7.5) mixed at a 92:2:2 ratio with methanol and tetrahydrofuran respectively. Buffer B consisted of methanol mixed with H_2O at a 65:35 ratio (47). Measurements were made using the fluorescence wavelengths of 334 nm for excitation and 425 nm for emission. Concentrations of Met and cystathionine were calculated by comparing their peak area to that of the internal standard.

5.2.5. Measurement of Homocysteine: Total plasma and media Hcy and Cys concentrations were determined using reverse phase HPLC and fluorescence detection of ammonium 7-fluoro 2 oxa-1,3-diazole-4-sulfonate thiol adducts as outlined by a previously published method (48).

5.2.6 Enzyme Assays: Lori Stead performed all the enzyme assays. The livers sent were thawed, and then diluted [5:1 (v/w)] in ice-cold 50 mM potassium phosphate buffer, pH 6.9. The livers were homogenized with a Polytron for 20 sec at 50% output and the homogenates were centrifuged at 18 000 g for 30 min at 4°C. All enzymatic assays were performed on the postmitochondrial supernatant (39).

CBS activity was measured using a previously published method (49). The assay mixture consisted of 16.6 mM Ser, 0.83 mM EDTA, 0.42 mM pyridoxal 5-phosphate, 33 mM D, L-homocysteine and 0.42 mM propargylglycine in 1M Tris buffer, pH 8.4. The reaction was started with the addition of 300 µg liver protein, and the mixture was incubated for 30 min at 37°C. The reaction was stopped with the addition of 100 µl, 10% SSA and then each sample was centrifuged for 5 min. at 14 000 g. The concentration of cystathionine in the supernatant was determined by HPLC as described in section 5.2.4.

AdoMet synthetase activity was measured using the protocol developed by Mudd *et al.* (50). Briefly, 133 mM Tris-HCl (pH 7.6), 177 mM KCl, 266 mM MgCl₂, 16 mM ATP, 3.5 mM glutathione, 0.26 mM AdoMet, 0.131 mM [¹⁴CH₃]-Met (150 x 10³ dpm) assay mixture was incubated for 30 min at 37°C with 750 µg liver cytosolic protein (total volume 450 µl). Ice-cold H₂O to a volume of 11.5 ml was added to stop the reaction. A 5 ml aliquot was passed through a Dowex 50-x4 (NH₄⁺) column (0.9 x 2.0 cm). The column was rinsed with 10 ml of H₂O and the radioactive AdoMet was eluted with 10 ml, 3 N NaOH. An aliquot of this elutant was measured for radioactivity and compared to the blank (measurement taken with heat-inactivated enzyme).

Met synthetase activity was assayed as previously described (51). The reaction mixture contained 87.5 mM potassium phosphate buffer, pH 7.5, 7.5 mM Hcy, 29 mM DTT, 0.25 mM AdoMet, 10 µM cyanocobalamin, 7 mM β-mercaptoethanol, 0.5 mM [5-¹⁴C] methyltetrahydrofolate (0.25 µCi) and 1.0 mg cytosolic protein. This reaction mixture was incubated for 30 min at 37°C. The incubation was performed in the dark, and the tubes were gassed with nitrogen and capped. The reaction was stopped with the addition of 0.5 ml ice cold H₂O, and the mixture was immediately passed through a Biorad AG1-X8 (200-400 mesh) column (Cl⁻). The column was rinsed 3x with 0.5 ml H₂O, the effluent was then added to 10 ml scintiverse and radioactivity was measured.

Methylenetetrahydrofolate reductase activity was determined using the method of Engbersen *et al.* (52). The reaction buffer contained 0.18 M potassium phosphate buffer (pH 6.8), 1.15 mM EDTA, 11.5 ascorbic acid, 54 μ M FAD, 20 μ M [^{14} C-CH $_3$] methyltetrahydrofolate (0.20 μ Ci), 3.5 mM menadione and 100 μ l cytosolic protein (150 μ g protein). The reaction mixtures were incubated in the dark at 37°C for 15 min. The reaction was stopped by the addition of 10 μ l of 1.0 M formaldehyde, 50 μ M dimedone in 200 μ l ethanol: H $_2$ O (1:1) and 100 μ l, 3.0 M potassium acetate pH 4.5. Next the samples were heated at 95°C for 15 min followed by a 10 min cooling period in an ice bath. Toluene (3 ml) was added to each sample followed by a 5 min centrifugation at low speed. Radioactivity was measured from 2.0 ml of the toluene phase.

Finally betaine: homocysteine methyltransferase activity was assayed using a modified version of a previously published method (53). Briefly, the reaction mixture consisted of 75 mM sodium phosphate (pH 7.4), 3.25 mM betaine, 10 mM D, L-Hcy and cytosolic protein (300 μ g). The assay proceeded over 15 min at 37°C and the reactions were stopped by adding 100 μ l of 10% SSA. The samples were stored on ice for 10 min and then centrifuged at 14 000 g for 5 min. Met was measured from the supernatant by reverse phase HPLC as described in section 5.2.4.

5.3 Results

5.3.1 Plasma Hcy Concentrations are Decreased in *Pemt*^{-/-} Mice: Female and male, *Pemt*^{-/-} and ^{+/+} mice were fed either a chow or HF/HC diet for 3 weeks as described in Chapter 2. Gender and diet were included as variables in this study since we had demonstrated previously that they affect the dependency on hepatic PEMT activity. Plasma was isolated from these animals and quickly frozen at -70°C. When the plasma samples for all animals had been collected, they were shipped for analysis to Memorial University (in dry ice). Interestingly, regardless of diet or gender, all the *Pemt*^{-/-} mice demonstrated an approximate 50% decrease in plasma Hcy levels compared to *Pemt*^{+/+} mice (Fig. 5.1). Unexpectedly, neither diet nor gender significantly affected the plasma Hcy concentrations. Plasma Hcy levels are affected by both diet and gender in humans. People who maintain a healthy diet generally have lower plasma Hcy than individuals

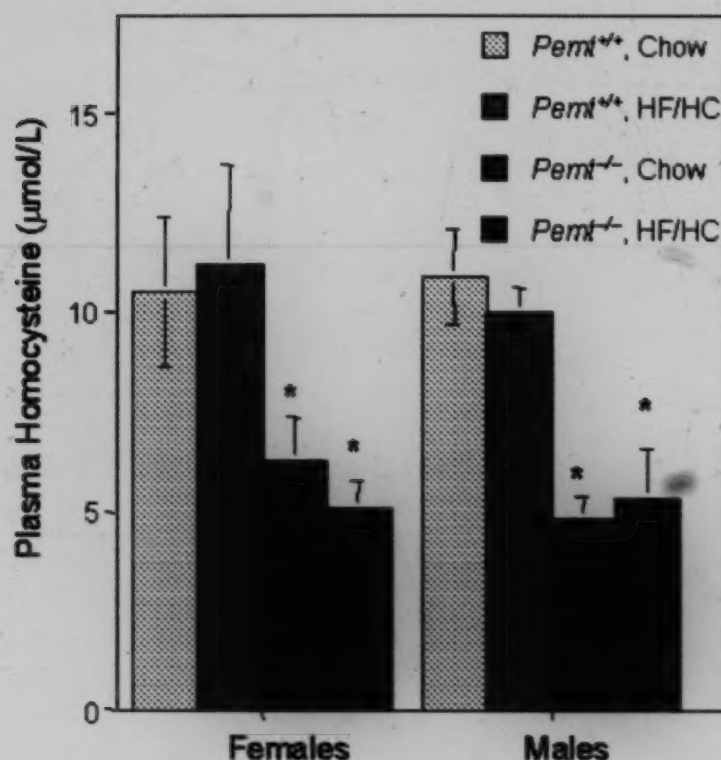


Figure 5.1: Plasma Hcy Levels are Diminished in *Pemt*^{-/-} Mice

Plasma Hcy levels were measured from plasma of *Pemt*^{-/-} and *+/+* animals fed either rodent chow or a HF/HC diet. Regardless of diet or gender, plasma Hcy levels were decreased by ~50% in all the *Pemt*^{-/-} mice. Shown is the mean of values obtained from 4-5 mice, $\pm$ S.D. * $P < 0.05$

who eat a diet enriched in fats and glucose (34). Furthermore, females normally have lower Hcy concentrations than males, however Hcy levels increase in females following menopause (54,55).

5.3.2 Loss of PEMT Activity is Responsible for Decreased Plasma Hcy: After the animals were sacrificed, their livers were removed and quick frozen in liquid N₂. Once all the livers had been collected they were transported to Dr. Brosnan's lab on dry ice. The livers were thawed and homogenized after which a number of enzymatic assays

were performed. These experiments were performed to confirm that the change in plasma Hcy levels were exclusively due to the loss of PEMT activity and not to changes in other enzymes involved in Met/Hcy metabolism. Activities for CBS, AdoMet Synthetase, Met Synthase, methylenetetrahydrofolate reductase and betaine:homocysteine methyltransferase were analyzed. No significant changes were observed between the *Pemt*^{-/-} mice compared to the ^{+/+} mice for any of enzymes assayed (Table 5.1A,B) confirming that the changes in plasma Hcy were likely due to a loss of PEMT activity.

5.3.3 Hepatocytes Isolated from *Pemt*^{-/-} Mice Secrete Less Homocysteine:

Next, we were interested as to whether or not the results observed *in vivo* could be repeated in cultured primary hepatocytes. Hepatocytes were isolated from male *Pemt*^{-/-} and ^{+/+} mice and were plated (2.0×10^6 cells/60 mm plate). After settling, the cells were rinsed and incubated for 18 hr in 1.5 ml of DMEM with 17% FBS, or serum free DMEM containing either 1% BSA /0.75 mM oleate, or 1% BSA alone. The conditions chosen (media volume and length of time) were very stringent in order to optimize the Hcy concentration in the media. Because the loss of PEMT activity significantly affected VLDL secretion only from the *Pemt*^{-/-} hepatocytes challenged with oleate (Chapter 4), we were interested as to whether or not that would affect Hcy secretion as well.

Hcy secretion was ~50% lower from the hepatocytes isolated from the *Pemt*^{-/-} animals compared to controls (Fig. 5.2). These differences were significant only in the cells plated in serum free media. Because serum contains a great deal of lipids, including LPC, it is possible that the hepatocytes isolated from the *Pemt*^{+/+} animals were less reliant on PEMT activity for total PC levels, thus less Hcy was produced by these cells. Unlike VLDL secretion, Hcy secretion was unaffected by the treatment of oleate. Nonetheless, these preliminary results confirm that PEMT activity contributes significantly to secreted Hcy pools. Only one experiment was performed since it was determined that the conditions chosen were too stringent.

Table 5.1: Specific Activities of Enzymes Involved in Methionine and Homocysteine Metabolism

Livers were removed from the sacrificed animals and quick frozen for transport. Prior to the enzymatic assays, each sample was homogenized and centrifuged. All assays were performed on a postmitochondrial supernatant. Shown are the means of values obtained from 3-5 animals, $\pm$ S.D. Data between groups was analyzed using ANOVA followed by Newman-Keuls post-test but no significant differences were found. *A.* Represents the values obtained from the male mice and *B.* represents the female values.

A. Males

nmole/mg/min	<i>Pemt</i> ^{-/-}		<i>Pemt</i> ^{+/+}	
	HF/HC	Chow	HF/HC	Chow
Cystathionine β -synthase	10.4 $\pm$ 0.7	11.1 $\pm$ 2.4	10.9 $\pm$ 1.2	11.7 $\pm$ 2.4
AdoMet Synthase	1.1 $\pm$ 0.2	1.0 $\pm$ 0.1	1.2 $\pm$ 0.2	1.0 $\pm$ 0.3
Met Synthetase	0.0672 $\pm$ 0.0073	0.0632 $\pm$ 0.004	0.0674 $\pm$ 0.0075	0.0536 $\pm$ 0.0148
Methylenetetrahydrofolate reductase	0.0220 $\pm$ 0.0026	0.0274 $\pm$ 0.0062	0.0335 $\pm$ 0.0044	0.0309 $\pm$ 0.0041
Betaine: Hcy Methyltransferase	4.0 $\pm$ 0.5	3.7 $\pm$ 0.4	4.2 $\pm$ 0.3	3.9 $\pm$ 0.3

B. Females

nmole/mg/min	<i>Pemt</i> ^{-/-}		<i>Pemt</i> ^{+/+}	
	HF/HC	Chow	HF/HC	Chow
Cystathionine β -synthase	13.5 $\pm$ 1.5	14.3 $\pm$ 1.4	13.5 $\pm$ 2.7	18.1 $\pm$ 5.8
AdoMet Synthase	0.778 $\pm$ 0.223	0.757 $\pm$ 0.146	0.922 $\pm$ 0.121	0.766 $\pm$ 0.223
Met Synthetase	0.0292 $\pm$ 0.0207	0.0223 $\pm$ 0.0298	0.0261 $\pm$ 0.00532	0.0313 $\pm$ 0.00998
Methylenetetrahydrofolate reductase	0.0334 $\pm$ 0.00818	0.0326 $\pm$ 0.00561	0.0374 $\pm$ 0.00753	0.0468 $\pm$ 0.00973
Betaine: Hcy Methyltransferase	3.6 $\pm$ 0.6	4.1 $\pm$ 0.5	3.8 $\pm$ 0.3	3.9 $\pm$ 0.4

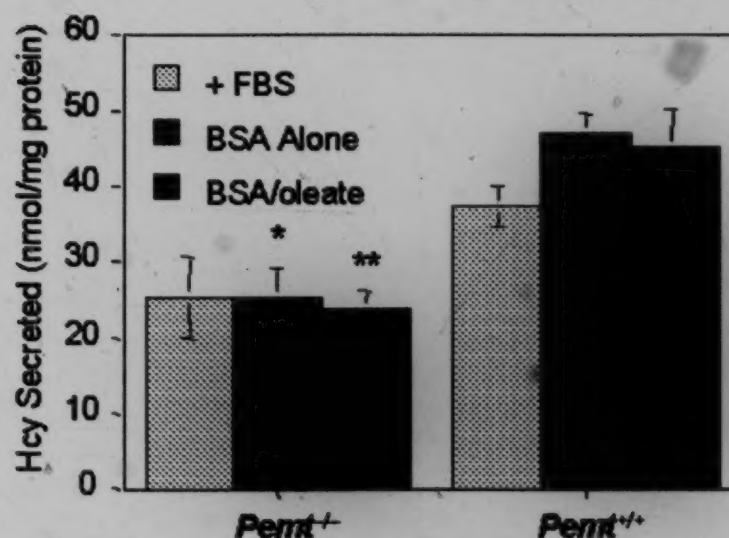


Figure 5.2: Hcy Export is Decreased from *Pemt*^{+/-} Hepatocytes

Hepatocytes isolated from *Pemt*^{+/-} and *+/+* mice and were plated and allowed to settle over a 5 hr period. The cultures were then incubated for 18 hr in either DMEM with 17% FBS, or with 1% BSA alone or in the presence of 0.75 mM oleate (without serum). After the 18 hr, the media was frozen and later analyzed for Hcy content. This experiment was performed only once, with triplicate plates for each condition. Shown is the mean of values, $\pm$ S.D. * $P < 0.01$ and ** $P < 0.05$ based on a paired Student's t-test.

A second pilot study was performed with the hepatocytes to try and determine more favorable conditions for this experiment. For such studies it is necessary to confirm that Met and Cys concentrations are unchanged. These measurements are important controls to verify that the changes in Hcy secretion are only due to the loss of PEMT activity. In the above study, these two amino acids were completely depleted by the end of the 18 hr incubation. Therefore in this experiment we tested various timepoints and quantities of media to determine optimal conditions for our study. Again, we determined that PEMT activity contributes significantly to secreted Hcy concentrations (Fig. 5.3). Met and Cys measurements on these samples are still in the process of being determined.

In this second experiment, we treated some of the plates with 200 μ M guanidinoacetate as a control (Fig. 5.3). Guanidinoacetate is a methyl acceptor and a

precursor for creatine. Methylation of guanidinoacetate has been verified to be an important contributor to exported Hcy pools (56). In treating the hepatocytes with this molecule we would expect to see an additional increase in Hcy secretion compared to control plates. Observing an increase in Hcy secretion would confirm that AdoMet concentrations are ample for additional methyltransferase reactions. This is an important

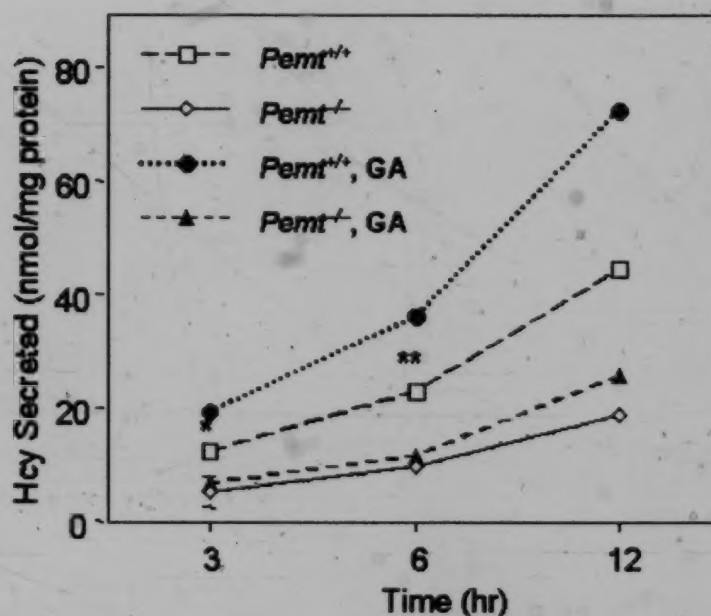


Figure 5.3: Pilot Study #2, Hcy Secretion is Diminished from *Pemt*^{-/-} Hepatocytes

Hepatocytes were isolated from *Pemt*^{-/-} and ^{+/+} mice and allowed to settle for 7 hr. The plates were rinsed and incubated in either 1.5, 2 or 3 ml of serum free DMEM for the indicated times (excluding 12 hr time point for 1.5 ml DMEM). At each timepoint, the cells and media were collected and frozen. The media was analyzed for Hcy and normalized to cellular protein. Shown is the average of all the range of media volumes for each time point, $\pm$ S.D. * $P < 0.01$ and ** $P < 0.05$ based on a paired Student's t-test comparing the *Pemt*^{-/-} and ^{+/+} values (triplicate plates for each time point). Only one plate for each time point was treated with 3 ml DMEM containing 200 μ M guanidinoacetate (GA) and the results obtained are as indicated. This experiment was performed only once.

measurement to make in order to confirm that the changes in Hcy secretion are not due to a depletion of intracellular AdoMet stores. Hcy secretion increased for both *Pemt*^{-/-} and *Pemt*^{+/-} hepatocytes in the presence of guanidinoacetate as compared to controls. The increase was ample for the *Pemt*^{+/-} hepatocytes but minor for the cells isolated from the *Pemt*^{-/-} mouse. It is noteworthy that the measurements were from only one plate of cells, isolated from one animal and so this experiment must be repeated. Nevertheless, this preliminary study again demonstrates that PEMT activity contributes significantly to Hcy export from primary hepatocyte cultures.

5.4 Discussion

In these experiments we have investigated the role of PEMT activity in regulating plasma Hcy levels. *In vivo* diet experiments confirmed that PEMT activity contributes up to 50% of total plasma Hcy levels in mice (Fig. 5.1). Interestingly, no differences were observed with regards to either gender or diet. In humans, male plasma total Hcy concentrations are consistently higher than females (54, 57). One study has demonstrated that plasma Hcy levels correlate positively with testosterone and inversely with estradiol concentrations (57). Hormone replacement therapy has also been demonstrated as an effective means of lowering plasma Hcy levels in peri- (55) and post-menopausal women (58, 59). The mechanism for the effect of estradiol on plasma Hcy levels has not yet been fully elucidated. One human study suggests that females secrete equal quantities of Hcy but metabolize it more quickly via the remethylation pathway (54). Additional studies are necessary however.

Furthermore, our observation that female mice rely more on PEMT activity than males (Chapter 3) also does not agree with the results obtained. If PEMT does play an important role in regulating plasma Hcy levels in mice (especially with the chow dietary condition) we should have observed elevated plasma Hcy concentrations in the female *Pemt*^{+/-} mice compared to the males. The results obtained in this study are however preliminary, and we have not taken into account a number of variables such as Hcy metabolism in the kidney.

Recent studies in humans also suggest that a typical Western diet enriched in saturated fats and glucose results in elevated plasma Hcy levels (34, 36). The mechanism for dietary mild hyperhomocysteinemia is not well understood. In our mice, loss of PEMT activity was more detrimental to plasma lipid levels in the animals fed a HF/HC diet, suggesting perhaps endogenous PEMT activity is elevated under these dietary conditions. Yet, no significant changes in total Hcy concentrations were observed in response to changes in diet. One possible explanation for our results is that the threeweek diet is not sufficient to elevate plasma Hcy levels significantly. As well, it is possible that the diet chosen for these experiments was inappropriate. Increasing evidence in both animals and humans verify the importance of insulin in the regulation of plasma Hcy levels (33, 36-38, 60). As mentioned previously (Chapter 1), little is known about the regulation of PEMT activity (61). Perhaps insulin regulates PEMT activity, since it is also known to regulate VLDL secretion. Additional studies in the *Pemt*^{-/-} and ^{+/+} mice should concentrate on long-term diet studies that include elevated sucrose or fructose intake.

Enzymatic assays in the livers of the animals tested, confirmed that loss of PEMT activity alone was probably the reason for the diminished plasma Hcy concentrations observed in the *Pemt*^{-/-} mice (Table 5.1). In addition, 2 pilot experiments performed on hepatocytes isolated from *Pemt*^{-/-} and ^{+/+} animals also confirmed that PEMT activity contributes significantly to secreted Hcy pools (Fig. 5.2, 5.3). A small increase in Hcy secretion with guanidinoacetate treatment suggests that the decrease in Hcy secretion observed from the *Pemt*^{-/-} hepatocytes was not a result of depleted AdoMet stores. This experiment was however performed only once and this result must be confirmed with additional experiments. This indirect method for looking at intracellular AdoMet levels in the hepatocytes was necessary because AdoMet is a very unstable molecule (62) and would not likely survive a freeze/thaw and analysis procedure. Lastly, our lab has obtained additional evidence concerning PEMT activity and its contribution to Hcy secretion. McArdle RH7777 cells stably transfected with human PEMT secrete significantly more Hcy than cells transfected with the vector alone (Dr. Yang Zhao *et al.*, unpublished results).

The idea that PEMT activity plays a role in regulating Hcy as well as AdoMet/AdoHcy metabolism is not entirely new. Vascular damage and atherosclerosis similar to that observed in homocysturmic patients can be induced in rats by feeding them a long term CD diet (63). As well, increased plasma Hcy and hepatic folate depletion are observed in rats fed a CD diet (64). Of course, under these dietary conditions PEMT serves as the primary source of PC and thus activity is elevated. Humans fed a choline/folate depleted diet demonstrated elevated plasma Hcy, as well as diminished plasma PC and folate concentrations (65). Interestingly, repletion of folate, but not choline into the diets restored plasma PC levels but did not decrease Hcy levels. Choline in the liver is oxidized to betaine, which serves as a methyl donor to Hcy for Met biosynthesis (4, 5). Therefore, in these studies the elevated plasma Hcy levels were primarily attributed to the loss of this remethylation pathway.

In conclusion, the results obtained from these preliminary experiments are the first to directly implicate PEMT activity and its contribution to basal plasma Hcy concentrations. In hindsight, these results are not necessarily surprising. Studies in rats suggest that PEMT can contribute up to 40% of the total PC produced by the liver (66). Bearing in mind the quantity of PC produced by the liver to maintain cellular integrity, provide structures for TG storage, maintain bile secretion as well as lipoprotein secretion implies that PEMT is responsible for a great deal of phospholipid biosynthesis. Furthermore, considering that 3 AdoHcy molecules are produced with each reaction completed by the PEMT enzyme, verifies that this pathway could contribute significantly to exported Hcy pools. However, now that it has been verified that PEMT contributes to plasma Hcy, further indepth studies need to be performed to determine if changes in PEMT activity contribute to the mild hyperhomocysteinemia observed in the presence of other cardiovascular disease risk factors such as diet, gender and age.

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Chapter 6

Summary and Future Directions

6.1 Role of PEMT Derived PC in the Secretion of VLDL

The original purpose of this thesis project was to study the role of PEMT derived PC with regards to VLDL secretion. In order to accomplish this task, we chose the *Pemt*^{-/-} mouse as a model for both *in vivo* diet studies (Chapter 2) as well as *in vitro* primary hepatocyte culture studies. This question is not a new topic of study since previous groups have attempted to answer it using non-specific, yet potent inhibitors of PEMT activity (1, 2). Unfortunately, the results from these studies were inconclusive, most likely due to the following reasons.

PC biosynthesis is essential for the existence of the cell, and for this reason there are a number of ways PC can be produced. Both the PEMT pathway and LPC reacylation support the primary PC biosynthetic Kennedy pathway (3). Furthermore, there are no less than 2 isoforms of each enzyme participating in the Kennedy pathway (4-6). Choline deficiency studies in animals and hepatocytes, as well as previous work in the *Pemt*^{-/-} mouse, have all demonstrated that with the loss of 1 PC biosynthetic pathway the other is activated in order to maintain PC homeostasis (7, 8). Thus, there is always a "backup" system for the loss of any component essential for PC biosynthesis. It is very possible that in the previous studies using the inhibitors, the Kennedy pathway was simply contributing to PC pools that normally would have been sustained by the PEMT pathway. As well, there are no known compounds that specifically inhibit PEMT activity. Therefore, in studies where a change in VLDL secretion was observed, it could never be wholly accounted for by the loss of PEMT activity (1, 2). Finally, even in those experiments where PEMT activity was diminished by greater than 95%, targeting of PEMT derived PC to VLDL secretion was still observed (9). Therefore, incomplete inhibition of PEMT could have also accounted for the differences in results observed by various groups.

For the above reasons, the *Pemt*^{-/-} mouse was chosen as the ideal model of study. Any changes in VLDL secretion would solely be a result of the complete loss of PEMT activity. As well, it has been previously demonstrated that CT activity is upregulated in

response to the loss of PEMT activity in these animals (8). In lieu of this fact, we could examine whether or not the Kennedy pathway was able to further compensate when presented with additional challenges to PC homeostasis, such as a HF/HC diet.

6.1.1 Hepatic TG Accumulation: In our studies outlined in Chapter 2, we demonstrated that PEMT activity contributed to VLDL secretion in a gender and diet specific manner. The only animals in which VLDL secretion was compromised were the male *Pemt*^{-/-} mice fed a HF/HC diet. This decrease in VLDL secretion was accompanied by a 2-fold increase in hepatic TG levels (Table 2.2). As well, extensive vacuolization was observed in the livers of these animals (Fig. 2.1).

Unexpectedly, these vacuoles did not stain with lipophilic Oil Red O, suggesting they are not lipid laden. This observation has been previously made in the *Pemt*^{-/-} mice fed a CD diet (C.J Walkey, unpublished results). To confirm our observations, these liver samples were further analyzed via imidazole staining and electron microscopy and again the vacuoles did not stain (Dr. M. Lee, unpublished results). However, imidazole staining requires the presence of polyunsaturated fatty acids and so one possible explanation is that the lipid accumulating within these vacuoles is of an unusual nature. There was some indication that some lipid leached during the fixative procedure. As well, only 1 liver sample for each condition was stained for lipid and therefore further examples should be analyzed to confirm our observations. These future analyses should incorporate more than one fixative and staining procedure in order to avoid the possibility that the experimental procedure itself affected the sample.

Another unusual feature of these hepatic vacuoles was their distribution. In our studies, we observed a pattern where vacuoles occurred within patches of hepatocytes, usually surrounding a blood vessel. It is noteworthy that there was only a 2-fold increase in hepatic TG levels suggesting the patterning observed was a result of only a mild elevation in hepatic TG. Still, similar patterning was also observed in 8 month old methionine adenosyltransferase 1A knockout mice fed a chow diet (10). Methionine adenosyltransferase 1A is the hepatic isoform of the enzyme that produces AdoMet and as a result, these mice had severely decreased levels of hepatic AdoMet (10). The fact that PEMT activity relies on AdoMet, and our studies indicate it also regulates Hcy

export in mice (Chapter 5), perhaps this unique form of vacuolization is linked to imbalances in Met metabolism. Nevertheless, it is clear that the vacuoles observed in these animals are different from those analyzed in many other genetically altered mice in which hepatic TG accumulation occurs and it would be interesting to determine the nature of these structures.

6.1.2 ApoB100 Versus ApoB48: The decrease in VLDL secretion in these male, HF/HC fed *Pemt*⁺ mice was limited to a loss of apoB100 secretion and not apoB48 (Fig. 2.5). As described in Chapter 1, the majority of evidence suggests that VLDL particles are synthesized via a 2-step mechanism (11). However, much of the experimentation performed on this model has concentrated on the synthesis of apoB48 containing lipoproteins (12). One study has suggested that the mechanisms for producing an apoB48 and an apoB100 particle differ (13). The observation that only apoB100 secretion was impaired in the *Pemt*⁺ males, and from hepatocytes isolated from these animals, concurs with this possibility. Perhaps, the assemblies of these two sets of particles occur in separate ER compartments utilizing different pools of lipid.

It is also possible that the loss of apoB100 secretion observed from these animals was simply due to a lack of lipid. It is well established that the apoB100 protein has much higher demands on lipid concentrations for its proper secretion compared to apoB48 (14). Studies examining these demands have focused primarily on the availability of TG but not on the availability of PC. According to the 2-step model for VLDL assembly, the apoB protein first assembles into a small, TG poor particle (11). This particle does contain phospholipid, which is necessary for the apoB protein to be properly folded. Taking into consideration that the apoB100 protein is twice the size of the apoB48 protein suggests that the demand for phospholipid at this first step would be higher. This hypothesis also takes into account why the defect in VLDL secretion occurred only in the presence of a high fat challenge. In the animals fed a chow diet, the demands on PC would be lower since bile and lipoprotein secretion would be normal. However, as a result of the increased demands for bile and lipoprotein secretion with the HF/HC diet, perhaps PC levels were depleted preventing normal apoB100 containing particle secretion.

Finally, one cannot ignore the recent results obtained from Asp *et al.* (15) where the bulk addition of lipid to the VLDL particles was verified to be ADP-ribosylation factor 1 and phospholipase D dependent. At the end of the publication, the authors suggested a number of hypotheses as to why phospholipase D activity would be necessary for the second step of VLDL assembly. One was that bulk TG addition occurred with the budding of a COP-1 vesicle. Conversion of the bilayer favoring PC molecule to the non-bilayer favoring phosphatidic acid and DAG has been shown on a number of occasions to play an important role in vesicular budding (16-18). Finally, there's the possibility outlined in detail in Chapter 4—that degradation of PC contributes to the mobilization of TG (15, 19). Regardless of which hypothesis is correct, the degradation of PC is important for the assembly and secretion of the VLDL particle. Where does this PC come from, and how large are the demands on this molecule for this function? Again, indepth analysis of intracellular apoB particles will hopefully shed light on this newly discovered role for PC.

6.1.3 PEMT Activity Regulates Plasma HDL Concentrations: VLDL secretion was not impaired in the female *Pemt*^{-/-} mice regardless of diet. Yet, for both diets there was a 30-40% decrease in plasma PC and cholesterol (Table 2.2, Fig. 2.2). Mice are HDL animals and so the majority of plasma PC and cholesterol would be expected to be in this fraction (20). HPLC analysis confirmed consistently in the HF/HC fed females, and inconsistently in the chow fed animals that the loss of these 2 lipids was indeed due to a loss in the HDL fraction (Fig. 2.3). As well, a smaller decrease in HDL derived PC and cholesterol was also observed in the male *Pemt*^{-/-} animals, but only in those fed a HF/HC diet (Figure 2.3).

The liver does not secrete HDL as a whole particle, but it is one of the 2 known organs to express and secrete apoA1. As well, ABCA-1 expression does occur in cultured rat hepatocytes (D. Sahoo *et al.*, unpublished results). Therefore, it is very possible that the liver contributes lipid to the HDL lipoprotein pool (Fig. 6.1). Little work has been done to elucidate the role of the liver with regards to HDL particle formation (21, 22). Such studies in the whole animal would be difficult since apoA1 can accept lipid from many tissues (23), and so to distinguish the quantity of lipid donated by

the liver to this fraction would be nearly impossible to determine. Still, preliminary data treating rat hepatocytes with free apoA1 have demonstrated that HDL particles can form via efflux of lipid from liver cells (D. Sahoo *et al.*, unpublished data).

SR-B1, a known HDL receptor is also expressed in the liver (24). It is well established that SR-B1 mediates selective uptake of cholesteryl ester from HDL and transfers it into the cell (24). Recently it was also demonstrated in cell culture that SR-B1 might mediate the transfer of phospholipids as well (25). In other steroidogenic tissues, SR-B1 specifically colocalizes with caveolae (26). Liver does not contain caveolae, indicating that perhaps regulation of this receptor differs here from other tissues (27). More specifically, it was demonstrated that hepatic SR-B1 is less sensitive to cholesterol storage depletion, whereas expression was greatly increased in the adrenal gland under these conditions (28). Finally, a number of studies have suggested that SR-B1 is regulated in a gender specific fashion (24, 28, 29).

Taken together, it is possible that the female *Pemt*^{-/-} mice are compensating for the loss of this PC biosynthetic pathway by regulating plasma HDL levels (Fig. 6.2). As described above, it is feasible that either loss of hepatic lipid to HDL by efflux is downregulated in these animals or perhaps lipid uptake via SR-B1 is upregulated. Preliminary studies examining SR-B1 expression in the livers of the diet study mice have so far been inconclusive. It would be possible to measure efflux of hepatic lipid to HDL particles *in vivo* by using a perfused liver model. Furthermore, one could devise an experiment to determine rates of labeled HDL phospholipid uptake into the liver, to see if they differ between the genders, and the *Pemt*^{-/-} and ^{+/+} animals.

Nonetheless, because the female *Pemt*^{-/-} mice are compensating for the loss of this PC biosynthetic pathway, even under normal dietary conditions, they are able to maintain VLDL secretion in the presence of the high fat challenge (Fig. 6.2). On the other hand, we have demonstrated that the male mice rely more heavily on the Kennedy pathway for PC biosynthesis (Chapter 3). As a result, the male *Pemt*^{-/-} mice produce sufficient PC even in the absence of PEMT activity under the normal dietary conditions so they do not require to modulate plasma HDL levels (Fig. 6.3). Because these male mice have not incorporated a compensatory mechanism beforehand, they are unable to tolerate any additional challenges on PC homeostasis (Fig. 6.3).

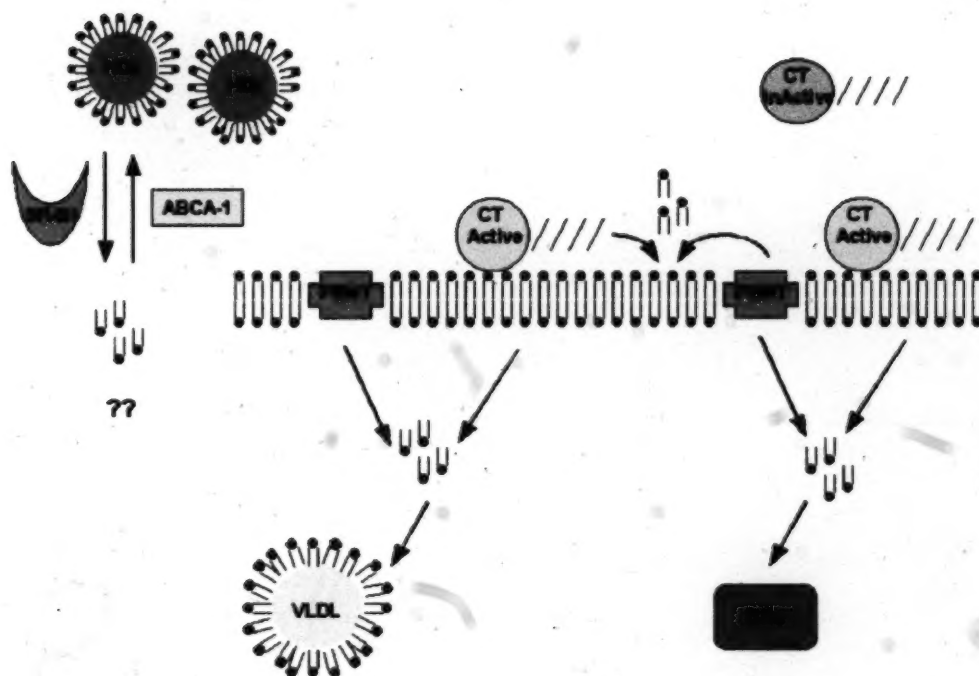


Figure 6.1: Summary of PC Metabolism in the Liver

PC is synthesized in the liver predominantly by 2 pathways. The major PC biosynthetic pathway is the Kennedy pathway, of which the rate limiting enzyme is CT. In its active form CT is associated with the bilayer, while in its inactive state it is soluble. The secondary biosynthetic pathway in the liver is catalyzed by PEMT. The PC produced by these two pathways has three known metabolic fates. It can be incorporated into membranes, targeted for bile secretion or for VLDL secretion. Evidence suggests that the liver can efflux PC to apoA1, through ABCA-1, to produce mature HDL particles. There is also the possibility that HDL contributes PC to the liver via the HDL receptor, SR-B1.

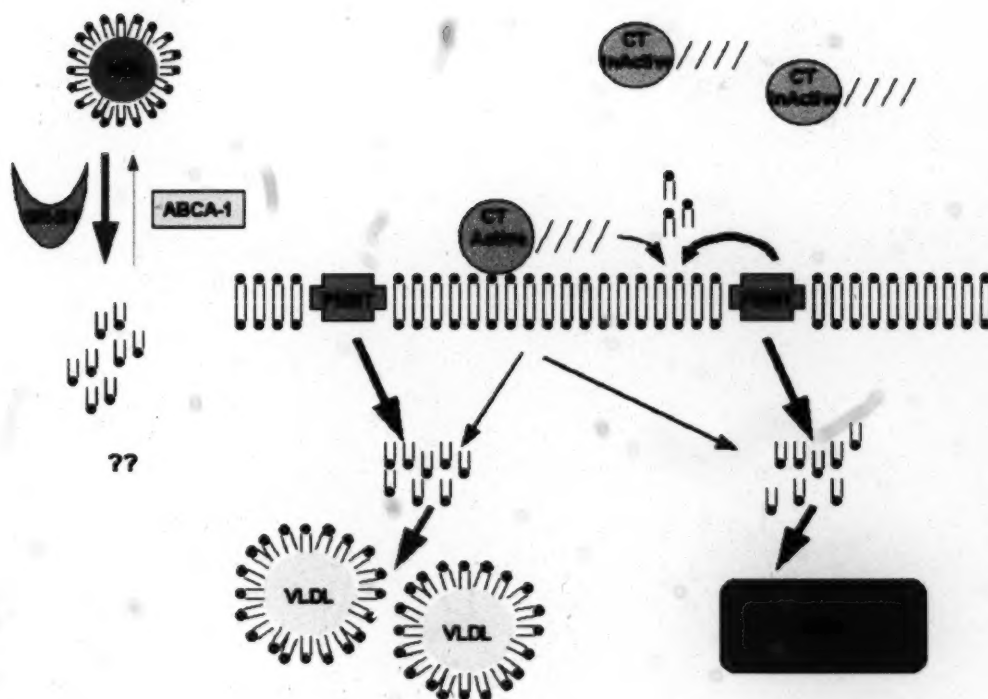


Figure 6.2: Hypothetical Model of PC Metabolism in Female Mice

Data obtained in our studies suggest that female mice rely more heavily on PEMT derived PC compared to males. As well, it has been demonstrated that in females, less CT activity is distributed to the active fraction. In the case of a HF/HC diet, more PC is directed to VLDL and bile secretion. In our *Pemt*^{-/-} females, we observed a decrease in plasma HDL. We hypothesize that the liver is compensating for the loss of PEMT activity in these animals by either decreasing efflux of PC to HDL particles or increasing uptake (as indicated by the red arrows). Because this compensatory mechanism is in place, the female *Pemt*^{-/-} mice have enough hepatic PC to maintain normal VLDL secretion (also indicated by a red arrow).

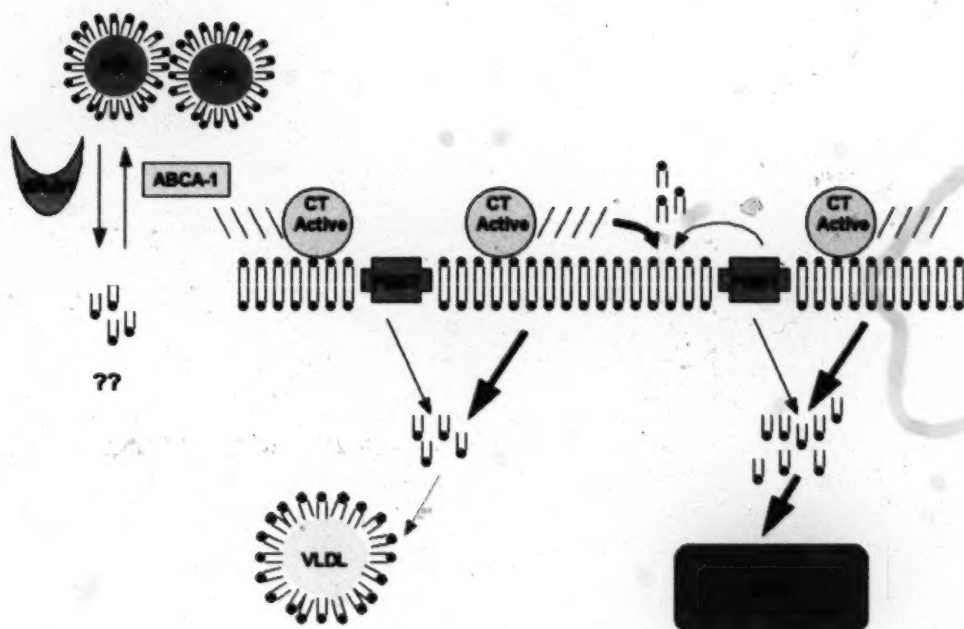


Figure 6.3: Hypothetical Model of PC Metabolism in Male Mice

Our analysis of CT activity in the livers of male mice suggests that they rely more on the Kennedy pathway for total PC biosynthesis. Because more CT is localized in the active fraction, the *Pemt*^{-/-} males do not recognize the loss of the secondary biosynthetic pathway (all effects with regards to the *Pemt*^{-/-} mice are indicated by the red arrows). As a consequence, plasma HDL levels remain normal under the chow dietary conditions. With the challenge of the HF/HC diet, cellular and biliary PC concentrations are maintained. However, because plasma HDL levels were not adjusted, hepatic PC pools are insufficient to maintain normal VLDL secretion.

6.1.4 VLDL Secretion from Hepatocytes: To confirm that PEMT activity is indeed important for VLDL secretion we also isolated primary hepatocyte cultures from *Pemt*^{-/-} and ^{+/+} male mice and compared TG and apoB secretion (Chapter 4). A 50% decrease in TG secretion (Fig. 4.2) as well as a loss in the steady state secretion of apoB100, with the oleate challenge, confirmed (Fig. 4.5) the results observed *in vivo*. The role of gender was not investigated using the cultured primary hepatocytes. As described in Chapter 4, the disadvantage of utilizing primary hepatocytes is that they lose a variety of functions over time, and they have a limited life span. Therefore, it is not known whether or not gender differences would come into play in these cultured hepatocytes. Furthermore, these hepatocytes are no longer under the influence of hormones that would normally circulate in the plasma.

The studies performed using cultured primary hepatocytes all utilized serum free media. These conditions were chosen in order to exclude the possible variable of serum lipids, which could influence the results observed. However, as described in section 6.1.3 these serum lipids may be an important source of PC for the *Pemt*^{-/-} animals (Fig. 6.2). It would be interesting to determine whether or not treatment with serum, HDL alone or LPC could rescue apoB100 secretion from the *Pemt*^{-/-} hepatocytes. If any of these results were positive, it would be an indication that the loss of VLDL secretion was a result of inadequate PC pools in the cell.

Primary hepatocytes could also be utilized to determine whether or not efflux of lipid to HDL particles is impaired in the *Pemt*^{-/-} animals. Separation of secreted radiolabeled PC into density fractions (Fig. 4.3) demonstrated no change in the HDL fractions of *Pemt*^{-/-} hepatocytes compared to the ^{+/+} cells. However, the particles found in these fractions are a mix of apoB48 and apoA1 containing particles. In order to study this hypothesis more fully, it would be necessary to directly measure the rate of lipid efflux to radiolabeled apoA1.

Because cultured hepatocytes are removed from the influences of other tissues and the various components of plasma, they are an ideal system to study the mechanism of VLDL secretion under controlled conditions (30). Since we repeated some of the observations made *in vivo*, in the hepatocytes, future studies should concentrate on utilizing this model to understand why these animals are impaired in their ability to

secrete apoB100 containing lipoproteins. Previous studies in CD rats, and hepatocytes isolated from CD rats indicated that when there is inadequate PC, VLDL particles are synthesized yet degraded in a post-ER compartment (31). It would be interesting to determine if that is the case in the *Pemt*^{-/-} male mice. Furthermore, the hepatocyte cultures could also be used to determine why apoB48 containing VLDL particle assembly is not impaired, while apoB100 is. Using these hepatocytes as a model may provide clues as to whether or not the assembly of the two types of VLDL particles differs.

6.1.5 Is PEMT Activity Required for VLDL Secretion?: Based on the observations made in both the *in vivo* diet studies, as well as in the primary hepatocyte cultures, it is clear that PEMT activity is not required for VLDL secretion. Female and male *Pemt*^{-/-} mice fed a chow diet, as well as the HF/HC fed females, did not display any impairment in their ability to secrete VLDL particles. Moreover, hepatocytes isolated from *Pemt*^{-/-} mice were capable of secreting normal levels of VLDL in the absence of oleate. In all of these cases, the upregulation of the Kennedy pathway (Chapter 3), as well as the effects on plasma HDL (in females) (Chapter 2), were sufficient changes to PC metabolism to prevent a loss of VLDL secretory function. These results do confirm that PC is required for regular VLDL secretion (Fig. 6.1). Activation of CT (Fig. 3.2) as well as the decreases observed in plasma HDL (Fig. 1.3) all imply that the liver was utilizing additional resources in order to compensate for the loss of PEMT activity. These results verify an already well-established fact, demonstrated by Yao and Vance in the late 1980s (32, 33).

Still the question remains, why were the male mice unable to tolerate the HF/HC diet? The observation that the male *Pemt*^{-/-} mice were not adjusting their plasma HDL levels under the chow dietary conditions, in contrast to the females, provides us with one clue (Table 2.4, Fig. 2.1). As well, previous observations in rats where males were shown to be more reliant on the Kennedy pathway compared to females also provides information as to what could be occurring in these animals (34, 35). If female mice are indeed more reliant on the PC produced by the PEMT pathway, it is possible that the loss of PEMT activity was more detrimental to the female mice as compared to the males. In order to repair the damage, the female *Pemt*^{-/-} animals adjusted the distribution of PC

away from the HDL fraction under the chow dietary condition (Fig. 6.2). On the other hand, the male mice did not seem to require adjusting PC distribution in response to the loss of PEMT activity under the normal dietary condition (Fig. 6.3). Because this additional compensatory pathway was not already in place, these animals were unable to tolerate the additional demands on PC with the HF/HC diet.

If the inability to upregulate VLDL secretion in response to the HF/HC diet was simply due to a lack of PC, why did the male mice not simply stimulate the Kennedy pathway further? As described in Chapter 3, the male mice, especially the *Pemt*^{-/-} male mice displayed a much higher percentage of total CT localized in the active microsomal fraction compared to the females (Fig. 3.2). One possibility is that of the CT protein available in these mice, it was saturated at the active fraction. Previous experiments in CD rats verified that long-term choline deficiency (3 weeks) was required before the liver recognized the loss of the Kennedy pathway and stimulated the additional expression of PEMT (7). Total CT activity was also elevated after 3 weeks of choline deficiency. Other studies examining CT activity during short-term choline deficiency (2 days) have only demonstrated a transfer of CT protein from the inactive fraction to the active (36). No change in total CT activity was observed in these experiments. Unfortunately, indepth studies examining the time line for activation of CT expression in response to a CD diet have not yet been performed.

Nevertheless, choline deficiency is a much more demanding challenge on PC metabolism than a HF/HC diet, yet there was no response at the level of PEMT transcription for an entire 3 weeks (7). Maybe the depletion of PC occurring in these male *Pemt*^{-/-}, HF/HC fed mice was insufficient to enhance any changes at the level of transcription for CT. It would be interesting to subject these *Pemt*^{-/-} males to long-term dietary studies to see if the status of the liver would become worse, or if some compensatory mechanism would finally be initiated. It is noteworthy that a 20% loss in total plasma PC and cholesterol was observed in the male *Pemt*^{-/-} mice fed the HF/HC diet (Table 2.4) and HPLC analysis demonstrated that part of this loss occurred in the HDL fraction (Fig. 2.3). Perhaps a longer exposure to this dietary condition would result in a further decrease in HDL levels, whereas apoB100 secretion would be rescued.

Although the diet and hepatocyte studies indicate that PEMT derived PC is not necessary for VLDL secretion, they do not address the possibility that this pool of PC is targeted for lipoprotein secretion. Even though other sources of PC can compensate for the loss of a PC biosynthetic pathway, this does not exclude the prospect that under normal conditions, newly synthesized pools of PC are targeted for specific roles within the cell. We attempted to address this problem in our studies outlined in Chapter 3. *Pemt*^{+/+} mice were injected with [³H]-Met and the distribution of radiolabeled PC amongst liver, bile and plasma was investigated. Our preliminary results suggested that PEMT derived PC is not specifically targeted for VLDL secretion as compared to any other source of PC. Still, these were the results of only one experiment and additional work should be performed to address this question.

6.1.6 A Matter of Priorities: No significant changes were observed in total liver and biliary PC for (Table 2.2, 2.3) the *Pemt*^{-/-} mice, regardless of diet or gender. Only the PC concentrations in plasma were affected with the loss of PEMT activity (Table 2.4). Interestingly, in rats fed a long-term CD diet (3-12 weeks), hepatic and other tissue PC concentrations remain normal. The livers remain enlarged and fatty suggesting that plasma lipoprotein and PC levels are affected by the decrease in PC biosynthesis from the Kennedy pathway (7). The observations made in the *Pemt*^{-/-} mice further suggest that in terms of PC distribution, lipoproteins seem to be of least importance.

Inadequate biosynthesis of PC in the cell is known to stimulate apoptosis (37). Thus it is understandable that the rank of cellular integrity over lipoprotein secretion would come into play. Prioritizing PC distribution to biliary secretion is not as clear. The inability to secrete bile due to a loss of the multi-drug resistance protein 2 has been verified to be extremely detrimental to liver function in mice (38). As well, lipids are an extremely important source of energy for the body (39). Malabsorption of lipids and lipid soluble vitamins would more than likely be much more harmful than the effects of a mildly fatty liver. Of course, bile is required for the proper emulsification and uptake of dietary lipids. Only in the last century and only in developed countries has excessive dietary fat intake become more of a problem than starvation. Of course, organisms are

designed to enhance their chances of survival. Therefore, it is likely that the ability to digest and absorb high-energy dietary fats is of more importance than VLDL secretion.

6.2 Regulation of PC Metabolism as a Function of Gender and Diet

Because of the gender specific differences observed in our *in vivo* diet studies, we were curious to understand the mechanism of these changes. As mentioned before, previous studies in rats implied that females rely more on PEMT derived PC than males (34, 35, 40, 41) (Fig. 6.2, 6.3). Because the HF/HC diet seemed to increase demands on PC levels in the *Pemt*⁺ animals, we were further intrigued as to the regulation of PC metabolism with regards to diet. Since VLDL secretion requires adequate concentrations of PC, and elevation of fat in the diet enhances the rate of VLDL secretion, we were interested in determining if either of the 2 primary PC biosynthetic pathways was elevated in response to diet.

6.2.1 *In vitro* PEMT and CT Assays: Enzymatic assays for PEMT and CT were performed on liver homogenates in the presence of saturating quantities of substrate. No changes in specific PEMT activity were observed in any of the *Pemt*^{+/+} animals tested, regardless of diet or gender (Fig. 3.1). This result suggests that neither gender nor diet regulate the total mass of PEMT protein available in the livers of these animals.

Previous studies performed by Ridgway *et al.* (42) demonstrated that in rat hepatocytes, there is excessive PEMT protein in relation to the availability of PE substrate. After a 2 day CD diet, PEMT activity was increased 2 fold when endogenous PE was used as the substrate. However, assays performed on the same samples using saturating quantities of exogenous substrate displayed no change in total activity. As well, Western blots confirmed that there was no change in enzymatic mass (using an antibody developed against purified PEMT). It was concluded that the increase in activity observed was due to an increase in available substrate as a result of a decrease in PC/PE ratios (42). Later, studies demonstrated that long-term choline deficiency was required in order to change total PEMT mass (7). In light of these results, it is safe to say that even though total PEMT activity was unchanged in response to diet or gender, this

does not rule out the possibility that *in vivo*, activity did not respond to these 2 variables. Furthermore, it seems that extremely harsh conditions such as long-term choline deficiency, is a requirement for any transcriptional activation of PEMT. Hence, perhaps the 3 week HF/HC diet did not impose sufficient demands on PC levels in the liver to induce a change in PEMT mass in these mice.

Total CT activity was mildly elevated in the *Pemt*^{-/-} animals compared to their ^{+/+} counterparts, however only in the chow fed mice (Fig. 3.2). The most striking observations were made with regards to the distribution of CT activity. As expected, a greater percentage of total CT activity was partitioned to the active, microsomal fraction in the *Pemt*^{-/-} mice compared to the ^{+/+} animals. Unexpectedly, regardless of diet or genotype, a large increase in activated CT levels was observed in the male mice compared to the females. This result implies that male mice do rely more heavily on the Kennedy pathway than females, concurring with results obtained in rats (Fig. 6.3).

It is unknown why male mice would preferentially utilize PC produced via the Kennedy pathway over PEMT derived PC. Our measurements performed in Chapter 2 suggest that male mice and their liver mass are slightly larger than females (Table 2.1). As well, male mice have significantly higher demands on plasma PC (Table 2.4, Fig. 3.8). The Kennedy pathway is the more efficient pathway for PC biosynthesis since it is able to recycle the degradation products of PC (43). Still, pregnancy and lactation are known to be very demanding on both PC and choline levels, suggesting that females would require the ability to surge PC production on demand (44). Nonetheless, the reality that the 2 major PC biosynthetic pathways are regulated in a gender specific manner does imply that they play different roles in overall PC homeostasis. The possibility that different pools of newly synthesized phospholipid could be playing specific roles in the cell has frequently been a topic of interest, but a difficult one to demonstrate (45). Therefore, it may be worthwhile to continue investigating the mechanism of this gender specific regulation of the Kennedy pathway, as well as the targeting of PC produced by these 2 pathways in females and in males.

6.2.2 Intracellular Distribution of PEMT: Immunoblots of PEMT demonstrated that this enzyme is distributed in the cell in a gender specific manner. The antibody,

which is specific for the C-terminal 12 amino acids (46), cross-reacted approximately 2 fold higher with the liver homogenates of female mice compared to males. This result provides very preliminary evidence that in the female mice, more PEMT activity is distributed to the MAM compared to the males. As described in Chapter 3, further indepth studies are required to confirm this hypothesis.

The MAM is a unique extension of the ER (47), which is in close proximity with the mitochondria. The function of MAM has not been elucidated, but this fraction is enriched in a number of lipid biosynthetic enzymes (47). One hypothesis suggests that the MAM plays a role in the exchange of lipids between the ER and the mitochondria (48). Another hypothesis takes into account the enrichment of this fraction with MTP, diacylglycerol acyltransferase, acyl-CoA:cholesterol acyltransferase, TGH, and apoB containing lipoproteins (47, 49, 50). This hypothesis proposes that the MAM is a major site of lipoprotein assembly and perhaps the site of TG pool formation.

If indeed a greater proportion of PEMT activity is distributed to the MAM fraction in female mice, tracking the distribution of PEMT derived PC in these animals may provide clues as to the function of this particular fraction. The distribution of PEMT activity between the ER and the MAM has not been quantitatively determined between the sexes. Presently, there are no antibodies that cross-react with the PEMT protein localized in the ER fraction making this task quite difficult and time consuming. The alternative would be to perform subcellular fractionations of murine hepatic tissues, and assaying the ER and MAM fractions. The disadvantage of this proposal is the quantity of tissue that would be required to isolate a measurable amount of MAM. The other alternative would be to utilize the separation technique developed in our lab using a DEAE Sepharose column (A. Noga *et al.*, unpublished data).

As with the role of the Kennedy pathway in the male mice, we do not understand the significance of this unique distribution of PEMT activity in the female mice. The results of our *in vivo* labeling study using [^{3} H]-Met imply that the female mice do utilize PEMT derived PC to a greater extent than males (Chapter 3). This result raises the possibility that PEMT activity can be regulated via its intracellular distribution. In rats, measurements of the PC/PE ratios in the MAM fraction resemble that of microsomes, and specific PEMT activity (using endogenous PE as a substrate) was also the same,

suggesting that the above proposal is unlikely (47). Nevertheless, the microsomes were not separated specifically into their subcellular fractions (rough ER, smooth ER, Golgi, etc...) and so this may not have been an adequate comparison of phospholipid and PEMT distribution. As well, these measurements were performed only in female rats and so it may be of interest to repeat these studies comparing males to females.

6.2.3 Targeting of PEMT Derived PC in Response to Gender and Diet: The purpose of the *in vivo* radiolabeling studies with [^3H]-Met was to determine whether or not PEMT derived PC was specifically targeted to any particular PC pool. One clear observation from this work was that with the HF/HC challenge, a great deal of PEMT derived PC was diverted away from the liver. As a result, there were very clear increases in the secretion of radiolabeled PC into the bile (Fig. 3.4, 3.5). Because the trends observed with regards to total PC mass mimicked what was observed for radiolabeled PC, specific activity was not necessarily affected. Therefore, PC secretion into bile in response to the HF/HC diet did not rely specifically on PEMT derived PC but significantly increased demands on total PC pools in the liver.

With regards to plasma PC, total mass increased in response to the HF/HC diet, and in the male mice compared to the females (Fig. 3.8). However, targeting of PEMT derived PC to the plasma was highest in the chow fed females, and lowest in the HF/HC fed males; results opposite to those observed for total PC mass. These trends with regards to targeting of PEMT derived PC to the plasma nonetheless were not significant. In addition, there were other trends observed in the liver and the bile in response to both the diet and gender that were also not significant. It is noteworthy that these analyses were performed on only 4-5 mice of each gender and diet, so it is hypothesized that many of these trends were deemed insignificant due to animal variability. The issue of variability is especially important since the *Pemt*^{-/-} and ^{+/+} colonies are still of a mixed background and have not yet been backcrossed to a single strain. Perhaps if the analysis had been extended to 7-10 animals for each variable, it is possible that these results would have been more pronounced. The variability also emphasizes the need to purify the strain of this colony before further studies are continued on these mice.

Regardless of the small number of animals studied, a significant change in the distribution of PC amongst the lipoprotein fractions was observed. Interestingly, in the male mice fed a HF/HC diet, a shift in total and radiolabeled PC occurred. The majority of secreted PC was transferred away from the VLDL fraction, to the HDL size density fractions (Fig. 3.6). The cause for this shift is unknown and contrasts with what was expected. No specific targeting of PEMT derived PC to any particular lipoprotein fraction was observed. As described in detail in Chapter 3, unfortunately the design of this experiment could not take into account the quantity of PC contributed to the HDL fraction by the liver and this aspect of PC homeostasis remains an unknown (Fig. 6.1).

Trends for specific activity in the liver, bile and the plasma all suggest that PEMT derived PC plays a greater role in female mice compared to males (Fig. 6.2). Not all of these trends were significant. Further investigation of the role of gender with regards to PC metabolism may provide insight into many unanswered questions such as the role of MAM, the role of the intracellular distribution of PEMT activity, as well as the roles of specific pools of newly synthesized PC.

6.3 PEMT Activity Contributes to Plasma Homocysteine Concentrations

PEMT is a methyltransferase that utilizes AdoMet as its methyl donor. With the development of the *Pemt*^{-/-} mouse, we now had an excellent model to study the contribution of PEMT activity to plasma Hcy levels. Our preliminary studies using the *Pemt*^{-/-} and ^{+/+} mice on the HF/HC and chow diets, demonstrated clearly that PEMT activity contributes up to 50% of total plasma Hcy in mice (Fig. 5.1). *In vitro* assays were performed on a number of other enzymes known to play important roles in either Met or Hcy metabolism. The results from these analyses validated that the differences observed in plasma Hcy were a direct result of the presence or absence of PEMT activity (Table 5.1). As well, 2 pilot studies with primary hepatocyte cultures isolated from the *Pemt*^{-/-} and ^{+/+} animals agreed with our *in vivo* measurements (Fig. 5.2, 5.3). Finally, additional experimentation utilizing PEMT expressing McArdle RH7777 hepatoma cells has further validated that PEMT activity contributes to the pool of Hcy exported out of the cell (Y. Zao *et al.*, unpublished results).

Normal Hcy concentrations in humans range from 5-16 $\mu\text{mol/L}$ of plasma though any concentration above 10 $\mu\text{mol/L}$ is considered harmful (51). As described in detail in Chapters 1 and 5, it is still not known whether mild hyperhomocysteinemia is a direct cause of cardiovascular disease (52). Since elevated Hcy concentrations occur usually along with other cardiovascular risk factors, there is some speculation that this toxic molecule merely enhances the rate of vascular blockage in people already at risk. There are others who suspect that mild hyperhomocysteinemia is simply a physiological indication of an unhealthy lifestyle (51, 52).

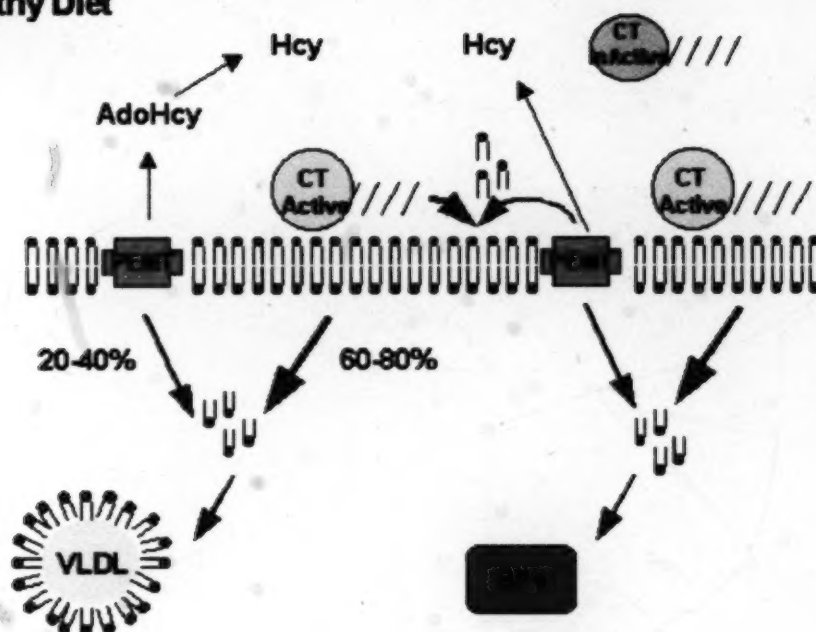
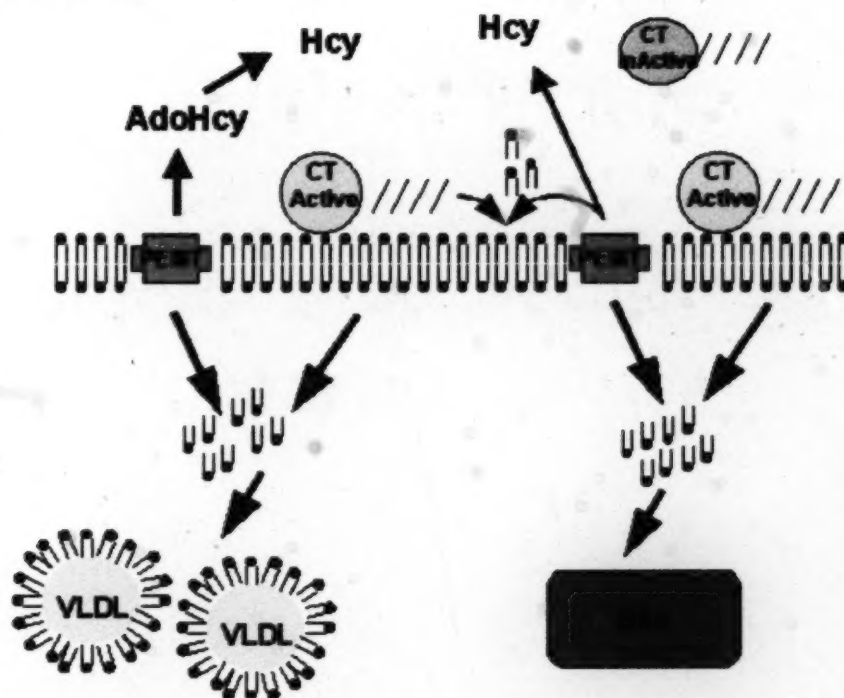
Our studies demonstrate that PEMT activity definitely contributes to basal plasma Hcy levels in the mouse. Nevertheless, we do not know if it contributes to elevated Hcy concentrations observed in the presence of other risk factors. In humans, with the consumption of a HF/HC diet (53), or in the case of insulin resistance (54), there is an overproduction of apoB100 containing particles. In addition, with the consumption of a HF/HC diet, one would expect increased demands on biliary PC secretion for proper digestion. Yet, what PC biosynthetic pathway is responsible for fulfilling these additional demands on PC homeostasis?

Our *in vivo* diet experiments suggest that the PEMT pathway provides the PC under these challenging conditions. The absence of PEMT activity in the *Pemt*^{-/-} mice resulted in either a change in plasma HDL, or VLDL, depending on the gender. As well, analysis of CT activity demonstrated that CT is not activated in response to the HF/HC diet (Fig. 3.2). Therefore, the results outlined in Chapter 2 and 3 suggest that the PEMT pathway alone provides the supplementary PC required beyond normal concentrations (at least with this HF/HC diet).

Based on the studies performed by Ridgway and Vance (42), we also know that even though there was no change observed in specific *in vitro* PEMT activity in response to diet (Fig. 3.1), this does not imply that endogenous *in vivo* activity is unchanged. With the increased secretion of PC to bile and VLDL (with a HF/HC diet), one would expect depletion in hepatic PC stores. If severe enough, this depletion could modulate the hepatic PC/PE ratios, which is known to be a sufficient stimulus to affect PEMT activity (Fig. 6.4). It is expected that this increase in PEMT activity in response to PC depletion would translate into increased Hcy production and secretion (Fig. 6.4B).

Figure 6.4: Hypothetical Model of PEMT Contributing to Hyperhomocysteinemia

A. In rats PEMT activity has been shown to contribute 20-40% of total hepatic PC pools. Therefore, the Kennedy pathway is the predominant biosynthetic pathway for PC in the liver. As shown in Fig. 6.1, PC can be distributed to the cellular membranes, VLDL or bile secretion. Our data demonstrates that in mice, PEMT activity contributes significantly to basal plasma Hcy pools. **B.** In the mice fed a HF/HC diet, no increase in activated CT was observed in response to the diet. The HF/HC diet increases demands on PC with the stimulation of both bile and VLDL secretion. Because more PC is leaving the liver (red phospholipids), there is a decrease in the PC/PE ratios (PE-green phospholipids). PEMT activity is elevated simply because there is more substrate available. In this way, PEMT compensates for the increased demands on PC levels with the high fat diet. Because PEMT activity is elevated, more AdoHcy and therefore more Hcy is produced. Over the long term, a HF/HC diet could perchance result in elevated plasma Hcy.

A. Healthy Diet**B. HF/HC Diet**

Taken together, we hypothesize that endogenous PEMT activity could potentially be elevated in response to any type of challenge on hepatic PC pools. Therefore, it could possibly contribute to the mildly elevated Hcy concentrations observed in patients at risk for cardiovascular disease. Nonetheless, a number of studies have already implicated changes in CBS and methylenetetrahydrofolate reductase activities in response to high glucose and insulin concentrations (55-58). Therefore, if PEMT activity does contribute to elevated Hcy levels, it does not do so alone.

In order to demonstrate this potentially toxic role of PEMT activity, the diet experiments performed in this thesis need to be extended beyond 3 weeks, since no changes in response to diet were observed in our experiments (Fig. 5.1). As well, it would be necessary to try other diets such as high carbohydrate diets, also known to greatly stimulate VLDL secretion and regulate CBS activity. To compliment these investigations, it would be very interesting to study PC metabolism and targeting, as well as plasma Hcy levels in either a diabetic mouse or rat model with hypertriglyceridemia.

6.4 PEMT Activity-A Risk Factor for Cardiovascular Disease?

Previous to the development of the *Pemt*^{-/-} mouse, the function of PEMT activity was unknown. Choline deficiency experiments in these mice demonstrated that PEMT activity was required as an alternative to the Kennedy pathway during times of choline restriction (59). Relevant physiological examples of choline deficiency are starvation (59), potentially pregnancy and lactation (44), as well as patients suffering from liver cirrhosis (60). However, we were interested in determining additional roles PEMT activity could be playing.

In this thesis, the focus was to determine the role of PEMT derived PC in the secretion of VLDL particles from the liver. In our studies, we have demonstrated that in mice, PEMT derived PC is involved in the secretion of apoB100 containing particles from the liver. However, this requirement for PEMT activity is only evident in the presence of a challenge on hepatic PC pools, in this case, a HF/HC diet. This effect on VLDL secretion was observed only in the male mice. Unexpectedly, loss of PEMT activity had a significant impact on plasma HDL levels, primarily in the female mice.

Beyond our original goal, we also determined that PEMT activity significantly contributes to plasma Hcy levels.

Gender, diet, lipoprotein metabolism and Hcy are all factors that play a role in the development of cardiovascular disease (Chapter 1). Unfortunately, how all these variables are linked to each other in order to result in the final outcome of the atherosclerotic lesion is not as well understood. It is well established that elevated levels of apoB100 containing lipoproteins contributes to the accumulation of lipid in the artery wall. It is also known that diet influences the concentration of plasma VLDL/LDL. HDL is protective against the development of the atherosclerotic lesion since this particle is capable of removing lipid from the vascular wall. Estrogen also appears to play a number of protective roles including lower LDL/HDL ratios. Finally, Hcy is a toxic molecule that has many deleterious effects.

Surprisingly, our results suggest that PEMT either influences, or is regulated by all of these factors. Elevated plasma VLDL/LDL levels as a result of a HF/HC diet seems to be at least partially dependent on PEMT activity and in this way PEMT activity contributes to hypertriglyceridemia. The increased requirement for PEMT activity in response to these dietary conditions could potentially increase plasma Hcy concentrations. The observation that gender influences the distribution and activation of both CT and PEMT activity suggests a potential mechanism for gender to regulate the secretion of lipoproteins and bile. Finally, PEMT activity influences either the production, or the turnover of plasma HDL. Overall, the results in this thesis project suggest that PEMT could have a stronger impact on the development of cardiovascular disease than previously thought. Still, many questions still remain unanswered and thus future studies should concentrate on understanding the mechanisms that link PEMT activity and PC metabolism to the described cardiovascular risk factors.

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